(12) NACH DEM VERTRAG ÜBER DIE INTERNATIONALE ZUSAMMENARBEIT AUF DEM GEBIET DES PATENTWESENS (PCT) VERÖFFENTLICHTE INTERNATIONALE ANMELDUNG

(19) Weltorganisation für geistiges Eigentum
Internationales Büro

(43) Internationales Veröffentlichungsdatum
2. November 2000 (02.11.2000)

PCT

(10) Internationale Veröffentlichungsnummer
WO 00/65073 A3

(51) Internationale Patentklassifikation[7]: C12N 15/82, 9/02, 5/10, A01H 5/00

(21) Internationales Aktenzeichen: PCT/DE00/01214

(22) Internationales Anmeldedatum: 14. April 2000 (14.04.2000)

(25) Einreichungssprache: Deutsch

(26) Veröffentlichungssprache: Deutsch

(30) Angaben zur Priorität:
199 18 365.1 22. April 1999 (22.04.1999) DE

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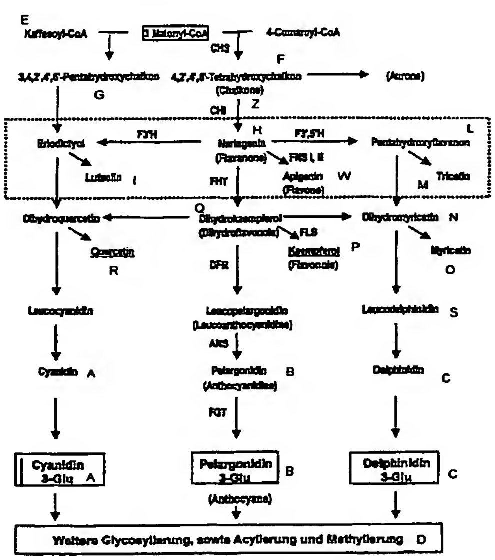
(81) Bestimmungsstaaten *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

[Fortsetzung auf der nächsten Seite]

(54) Title: GENETIC SEQUENCE WHICH CODES FOR THE FLAVON SYNTHASE II ENZYME AND USE OF THE SAME

(54) Bezeichnung: GENETISCHE SEQUENZ, DIE FÜR FLAVONSYNTHASE II ENZYME KODIERT, UND DEREN VERWENDUNG



A ... CYANIDINE
B ... PELARGONIDINE
C ... DELPHINIDINE
D ... ADDITIONAL GLYCOSYLATION, ACYLATION AND METHYLATION
E ... CAFEOYL-CoA
F ... TETRAHYDROXYCHALCONE
G ... PENTAHYDROXYCHALCONE
H ... NARINGENINE
I ... LUTEOLINE
L ... PENTAHYDROXYFLAVANONE
M ... TRICETINE
N ... DIHYDROMYRICETINE
O ... MYRICETINE
P ... CAMPHEROL
Q ... DIHYDROCAMPHEROL
R ... QUERCETINE
S ... LEUCODELPHINIDINE
W ... APIGENINE
Z ... CHALCONE

(57) Abstract: The invention relates to genetic sequences which code for the enzyme of the flavonoid metabolism, in particular, for flavon synthase II (FNS II) or derivates thereof and to their use for specifically modifying the colour of flowers, for modifying the flavon content or expression in leaves, flowers and other plant or organic tissue. The use also covers expression systems for synthesising natural, functional flavons for medical or similar applications, for example, for treating cancer or for improving the human immune defence.

(57) Zusammenfassung: Die vorliegende Erfindung bezieht sich auf genetische Sequenzen, die für Enzyme des Flavonoidstoffwechsels kodieren, speziell für die Flavonsynthase II (FNS II) oder Derivate hiervon und deren Verwendung zur gezielten Veränderung der Blütenfarbe, zur Veränderung des Flavongehaltes bzw. -musters in Blättern, Blüten und anderen Geweben von Pflanzen und anderen Organismen und darüberhinaus die Verwendung in Expressionssystemen zur Synthese von natürlichen, funktionellen Flavonen für medizinische oder ähnliche Anwendungen, z.B. zur Krebstherapie oder Verbesserung der menschlichen Immunabwehr.

WO 00/65073 A3

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Canadian Intellectual Property Office
An agency of Industry Canada

CA 2370387 A1 2000/11/02

(21) 2 370 387

(12) DEMANDE DE BREVET CANADIEN
CANADIAN PATENT APPLICATION

(13) A1

(86) Date de dépôt PCT/PCT Filing Date: 2000/04/14
(87) Date publication PCT/PCT Publication Date: 2000/11/02
(85) Entrée phase nationale/National Entry: 2001/10/19
(86) N° demande PCT/PCT Application No.: DE 2000/001214
(87) N° publication PCT/PCT Publication No.: 2000/065073
(30) Priorité/Priority: 1999/04/22 (199 18 365.1) DE

(51) Cl.Int.^{7}/Int.Cl.^{7} C12N 15/82, A01H 5/00, C12N 5/10, C12N 9/02
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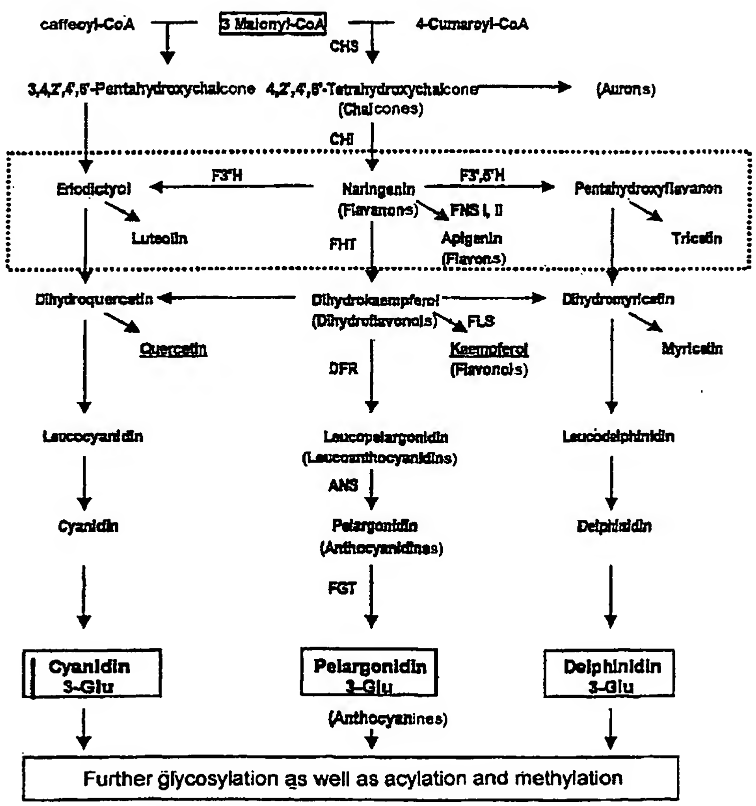
(54) Titre : SEQUENCE GENETIQUE CODANT POUR L'ENZYME FLAVONE II SYNTHASE ET SON UTILISATION
(54) Title: GENETIC SEQUENCE WHICH CODES FOR THE FLAVON SYNTHASE II ENZYME AND USE OF THE SAME



(57) Abrégé/Abstract:
The invention relates to genetic sequences which code for the enzyme of the flavonoid metabolism, in particular, for flavon synthase II (FNS II) or derivates thereof and to their use for specifically modifying the colour of flowers, for modifying the flavon

(57) **Abrégé(suite)/Abstract(continued):**
content or expression in leaves, flowers and other plant or organic tissue. The use also covers expression systems for synthesising natural, functional flavons for medical or similar applications, for example, for treating cancer or for improving the human immune defence.

GENETIC SEQUENCE WHICH CODES FOR THE FLAVONE SYNTHASE II ENZYME AND USE OF THE SAME

The present invention relates to genetic sequences encoding enzymes of the flavonoid metabolism, in particular flavone synthase II (FNS II) or derivatives thereof as well as to the use thereof for the targeted modification of the color of flowers, for altering the flavone content or the flavone pattern, respectively, in leaves, flowers and other tissues of plants and other organisms, moreover, it relates to the use in expression systems for the synthesis of natural, functional flavones for medical or similar applications, for example for the treatment of cancer or for improving the human immune defense.

Flavonoids and their function in plants

Flavonoids are the most important and most widespread plant pigments which have been detected in various tissues, such as flowers, leaves or roots. Furthermore, they are among the best characterized secondary metabolites in plants. Up to now, more than 3000 different flavonoids have been characterized. They have been divided into different subclasses (e.g. flavones, flavonols, or anthocyans) based on the degree of oxidation of the central C ring. Additionally, each type may be further modified by hydroxylation, acylation or glycosylation (Heller and Forkmann, 1994). Due to the different physico-chemical properties of the molecules, the subclasses may in part exhibit very different biological functions.

The accumulation of certain flavonoids in a plant cell depends on the availability of the corresponding enzymes wherein the availability of the enzymes ultimately is dependent on the expression of the respective gene. Regulation of the expression of the genes of flavonoid biosynthesis is substantially determined by the plant species, the developmental stage and environmental conditions.

Flavonoids play an important role both within and outside of the plant. Thus, for example, certain flavonols have been shown to be required for growth of the pollen tube. If the accumulation of flavonols is suppressed by blocking their biosynthetic pathway, sterile pollens are obtained (Taylor and Jorgensen, 1992). Both biotic and abiotic signals may result in an accumulation of flavonoids during

interaction of a plant with its environment. Thus, for example, UV irradiation leads to an accumulation of flavonols and flavones. This is achieved by induction of the transcription of the respective flavonoid biosynthesis genes in different species (Kubasek et al., 1992). But also other stress factors such as wounding, extreme temperature variations and water stress may induce flavonoid accumulation and/or gene expression in different species (Hradzina, 1982). Flavonoids have been assigned a double role in interactions of plants with other organisms. On the one hand, as phenolic compounds flavonoids have a phytoalexin effect against various pathogens and as a deterrent against predators (Harborne and Grayer, 1994), on the other hand they are responsible for the communication between plants of the family of *Leguminosae* and particular microorganisms. In this respect, flavonoids serve as signaling agents for nitrogen fixing bacteria which then express genes required for establishing a symbiosis with the plant (Redmond et al., 1986). In flowers, leaves, and fruits, the flavonoids and particularly the colored anthocyans but also chalcones, aurones, flavones, and flavonols, are responsible for the coloring and the patterns of various secondary metabolites. Together with other characteristics such as e.g. the scent the latter is important for recognition by various animals but also for humans using the plant as decoration or food stuff (Harborne and Grayer, 1994). Furthermore, particular flavonoids such as the flavone apigenin and the flavonol quercetin, have an effect on the auxin transport within a plant (Jacobs and Rubery, 1988)

The flavonoid biosynthesis pathway (Fig. 1A).

The structure of flavonoids includes two aromatic rings (A and B) and a central heterocycle (C) (Fig. 1B). Within the plant, they are synthesized starting from L-phenylalanine via the phenyl propanoid pathway by the enzymatic reaction of phenylalanine ammonia lyase (PAL) and cinnamonic acid 4-hydroxylase (4CL). 4-Cumaroyl CoA resulting from this reaction together with 3 molecules of malonyl CoA yields tetrahydroxychalcone. This reaction is catalyzed by chalcone synthase (CHS), the key enzyme of flavonoid biosynthesis (Fig. 1A). Generally, tetrahydroxychalcone (THC) is quickly isomerized to yield naringenin (NAR) by the enzyme chalcone isomerase (CHI). Different subsequent reactions generate the anthocyans. Flavones are formed via a side pathway by the action of FNS I or FNS II, a cytochrome P450 enzyme. This class of enzymes is widespread in nature, and

various genes for cytochrome P450 enzymes have been isolated and sequenced from vertebrates, insects, yeasts, fungi, bacteria, and plants.

Flavone synthase uses different flavanones such as NAR or eriodyctol (ERI) as substrates to synthesize the corresponding flavones apigenin (Ap) and luteolin (Lu). For this purpose, a double bond is introduced between positions C2 and C3, as shown in Figure 1B. Flavones may be present in plants in glycosylated as well as in methylated forms.

The formation of flavones from flavanones *in vitro* has been observed first in enzyme preparations of UV irradiated *Petroselium crispum* cell suspension cultures. The corresponding enzyme is a soluble 2-oxoglutarate-dependent dioxygenase which has been referred to as flavone synthase I (FNS I). In the flowers of various flavone-producing plants, e.g. *Sinningia cardinalis, Antirrhinum majus, Verbena hybrida, Columnea hybrida, Chrysanthemum morifolium, Gerbera hybrids* and osmotically induced cell suspension cultures of *Glycine max*, this reaction, however, is catalyzed by the above-mentioned FNS II, an NADPH-dependent microsomal enzyme belonging to the class of P450 cytochromes (Heller and Forkmann, 1994). The formation of flavones in flowers of *Gerbera hybrids* is regulated by the *Fns* locus. Flavone synthesis is only detected in lines which carry the dominant allele while no FNS II activity is detectable in homozygous recessive lines (Fig. 2; Martens and Forkmann, 1998).

Three types of pigments are responsible for the color in flowers: betalains, carotenoids, and flavonoids. Betalains are present only in a few families of the *Centrospermae* in which they are responsible for yellow, orange, red, and purple colors. Carotenoids result in orange and yellow shades and are the main pigments of most of the orange and yellow flowers. Flavonoids are the most important and most widespread pigments in flowers and plants, respectively. This group includes the coloring anthocyans which are present in the vacuole in glycosylated and often acylated forms. Different anthocyans are able to produce different shades. The color of flowers is further affected by the pH of the vacuole, the complexation with metals and the pattern of glycosylation and acylation, respectively (Forkmann, 1991). Another important factor for the generation of the various flower colors is copigmentation of anthocyans with colorless flavonoids, such as

flavones or flavonols, or also with tannins (Scott-Moncrieff, 1936). Anthocyans which are copigmented with flavones may attain different colors depending on the basic structure of the anthocyan which may vary between purple and blue (Asen and Horowitz, 1974; Goto and Kondo, 1991). Moreover, several flavones, such as isoetin, have been identified as yellow flower pigments (Harborne, 1978).

It is an important aim in horticultural plant breeding to develop novel varieties of flowering ornamental plants. In the past, classical methods of breeding have been partly successful in establishing a number of different colors in many economically important ornamental plants. The gene pool of the individual species, however, limits possibilities of such approaches carried out in a natural manner. This is the reason why today there are only a few species showing the whole spectrum of colors. Furthermore, the alteration generated by means of classical methods of breeding cannot be targeted. Since the aesthetic value of a flower is determined by various factors, such as form, scent and color, and an alteration of one of this factors by crossing generally can only be achieved to the expense of similar other, visible characteristics and is extremely lengthy and laborious, an effective way to achieve novel varieties must be utilized. The possibility of altering the color of plant flowers in a targeted manner provides clear advantages as compared to other methods. This is particularly important in an area with a high product turnover in which novelty is an important market factor. For example, the development of blue flowering varieties of the main cut flower species, such as roses, chrysanthemums, carnations, lilies, tulips, and gerbera would lead to a substantial market advantage on the cut flower market, but also on the potted plant market. The possibility of controlling the synthesis of copigments, e.g. flavones, in plants is a beneficial application of the targeted alteration of flower colors. In addition, besides flowers this application may also be applied to fruits and other agricultural plants, e.g. fruit and vegetable plants, and to leaves, e.g. of ornamental plants.

Besides their contribution to the color of flowers, the flavonoids and particularly the flavones also have other biological properties and effects. For example, in some plants they have been found to be a feed stimulant for monophagous and oligophagous insects (Harborne and Grayer, 1994). In most cases, the glycosides exhibit a higher effect than the corresponding aglycones presumably due to an

improved solubility of the glycosides. Furthermore, insects are able to distinguish between different sugar residues whereby the active components are further differentiated. In addition, also the basic structure of the aglycones may result in different effects. Compared to many other secondary plant metabolites, the flavonoids and flavones, respectively, obviously have no excessive toxic effect on insects. Nevertheless there are some flavones which already in very low concentrations are able to act as a deterrent for feed insects or to severely inhibit the growth of the animals. In this respect, no effect of the type of glycosylation could be demonstrated. However, an effect of the type of hydroxylation or methoxylation, respectively, of the flavone could be demonstrated (Harborne and Grayer, 1994).

Moreover, several flavones stimulate the egg laying of butterflies on specific plants. It has been shown that egg laying does not occur until the animals have recognized the stimulus. Such stimulating substances include for example flavones the vicenin-2 and various luteolin derivatives. If the synthesis of these substances is blocked in the respective host plants it is possible to inhibit egg laying by the butterflies and, thus, feed damages by their caterpillars.

Utilizing the natural chemical defense mechanisms of the plants may avoid the problems encountered with the use of synthetic insecticides such as e.g. environmental pollution caused by residues of the substances used in fruits and soil. In addition resistance generation observed with most of the synthetic pesticides is avoided or at least delayed, and the sometimes extensive costs for agents and applications are saved.

Another important biological property of the flavonoids relates to the activation of nodulation genes in various rhizobium species. These bacteria infect leguminous plants and form nitrogen-fixing root nodules. In this process, the flavonoids produced by the host plant act as a "signaling agent" whereby the bacteria induce the process of infection. These plant-specific active compounds also include various flavones such as apigenin, luteolin, and 7,4'-dihydroxyflavone (Firmin et al., 1986; Redmond et al., 1986). Altering the production and delivery of flavones by the root or the flavone pattern, respectively, in this tissue provides a possibility

to improve the nitrogen fixation and probably to establish this mechanism also in non-leguminous plants.

Utilizing this natural symbiotic mechanism may reduce nitrogen fertilization and thereby the environmental pollution by washing away of the nutrients. Moreover, the costs for fertilizers and for their spreading are saved.

Within a plant, several naturally occurring flavonoids such as the flavone apigenin or the flavonols kaempferol and quercitin affect the transport of auxins in different plant tissues and transport systems. In this respect, they act similar to chemical transport inhibitors. As growth regulators of plants the auxins themselves affect cell extension, cell division, apical dominance, reformation of roots and shoot as well as parthenocarpy. An induced, altered flavonoid concentration (endogenous change and/or exogenous application), thus, may have a significant effect on plant growth via their interaction with auxins. This may allow for a substitution of synthetic growth inhibitors.

Moreover, as bioactive substances flavonoids have a non-negligible role in the diet of men and animals. They are found in fruits, vegetables, nuts, seeds, shoots but also in tea and wine. Since some time, anti-allergic, anti-inflammatory, anti-viral, proliferation-reducing and anti-cancer properties are attributed to flavonoids and also to several flavones. But also an effect on the metabolism and the highly complex immune system of humans and animals has been described. In this respect, flavonoids and flavones, respectively, have an effect on a large number of different enzymes (e.g. on hyaluronidase or aldose reductase), they have important enzyme inducing and anti-oxidative properties, and are capable of scavenging free radicals, chelating several metal cations, and have an effect on cellular protein phosphorylation (Middleton and Kandaswami, 1994).

If agricultural plants important for the human and animal nutrition because of their content in health-promoting flavonoids can be improved by a targeted alteration of the content or pattern of the respective compounds this would greatly contribute to a healthy diet of men and animals.

Therefore, it has been an object of the present invention to provide means and methods to alter and control the flavonoid biosynthesis and the formation of flavones, respectively, in plants in a targeted manner in order to e.g. alter the color of a plant flower or to improve the resistance properties and capability to establish symbioses of a plant.

It has been another object of the present invention to provide means and methods useful for a targeted synthesis of defined flavones. Flavones obtained in such manner may find use i.a. in cancer therapy or may contribute to the health of men and animals in the form of medicaments.

According to the present invention, this object has been achieved by the claims.

Therefore, the present invention relates to a nucleic acid sequence encoding a flavone synthase II (FNS II). In one embodiment, the present invention relates to a nucleic acid sequence as shown in SEQ ID NO:1, or to a portion thereof. In another embodiment, the present invention relates to a nucleic acid sequence hybridizing to a nucleic acid sequence as shown in SEQ ID NO:1 or a portion thereof and/or having a homology of at least 40%, more preferably at least 45%, further preferred at least 55% or most preferably at least 65-70% or most preferably a homology of more than 85% on the level of the nucleic acid sequence or amino acid sequence to at least one or more regions (preferably to the whole region) of the sequence as shown in SEQ ID NO:1. Preferably, the nucleic acid sequence encodes a protein or a polypeptide having the biological activity of a flavone synthase. In another embodiment, the present invention relates to a nucleic acid sequence which is degenerated with respect to a nucleic acid sequence according to the above-mentioned embodiments. In a preferred embodiment, the nucleic acid sequence according to the present invention is DNA or RNA and is derived from a flavone-containing plant such as gerbera (*Gerbera hybrids*), aster (*Callistephus chinensis*), snapdragon (*Antirrhinium majus*), chrysanthemum (*Chrysanthemum indicum*), dahlia (*Dahlia hybrids*), gloxinia (*Sinningia hybrids*), verbena (*Verbena hybrids*), and *Streptocarpus* (*S. hybrids*). In another preferred embodiment the nucleic acid sequence according to the present invention is a recombinant nucleic acid sequence. Furthermore, the present invention relates to a

nucleic acid sequence complementary to the sequence encoding flavone synthase II (FNS II).

Accordingly, the present invention provides an isolated nucleic acid sequence comprising a nucleic acid sequence encoding flavone synthase II (FNS II) or a functional derivative of said enzyme or a nucleic acid sequence complementary thereto. The term "FNS II enzyme" means enzymes of the flavonoid biosynthetic pathway using flavanones such as naringenin and eriodictyol or also other compounds of this class as a substrate for the synthesis of the corresponding flavones.

Preferred is a nucleic acid according to the present invention which has been isolated from its natural environment or chemically synthesized. Particularly preferred are nucleic acid molecules which are formed or obtained *in vitro* including genomic DNA fragments, recombinant and synthetic molecules and nucleic acids in combination with heterologous nucleic acids. This also comprises genomic DNA or cDNA or portions thereof encoding FNS II or portions thereof in a reverse orientation to its own or another promoter. Further comprised are naturally occurring, closely related nucleic acid sequences.

The term "nucleic acid sequence encoding a flavone synthase II" is used herein in its most general form and comprises any sequential order of nucleotide bases defining, directly or via a complementary array of bases, an amino acid sequence of an FNS II.

A polypeptide having a portion or the complete amino acid sequence of flavone synthase II means a full length FNS II or an active incomplete form thereof.

In another embodiment, the present invention relates to oligonucleotides which may be used as genetic probes or "antisense" molecules for controlling the expression of the corresponding gene in plants or other organisms. An "antisense" molecule as described herein also comprises a gene construct consisting of a structural, genomic or cDNA gene or a portion thereof in reverse orientation with respect to its own or any other promoter.

In another embodiment, the nucleic acid sequence encoding FNS II or various functional derivatives thereof is used to reduce the activity of endogenous FNS II, or alternatively a nucleic acid sequence encoding said enzyme or various derivatives or portions thereof is used in antisense orientation to reduce the activity of FNS II. Moreover, it is also possible that an antisense transcript of FNS II or a fragment or a portion of FNS II (e.g. an oligonucleotide molecule) forms a duplex with the whole or portions of the naturally occurring mRNA which specifies the enzyme and thus inhibits an accumulation of or the translation of the mRNA into the active enzyme. Another possibility is the use of ribozymes to inactivate specific nucleic acid sequences.

Alterations of the FNS II activity mentioned herein relate to an increase or decrease of the activity of up to 30% or more preferably 30 to 50% or still more preferably 50 to 75% and most preferably of 75% or even higher or lower, respectively, as compared to the normal, endogenous or existing activity value. The amount of the activity can be tested easily using the method described in Martens and Forkmann (1998) (see Example 3).

The nucleic acid described in the present invention may also be a ribonucleic acid or a deoxyribonucleic acid existing in the form of a single stranded or double stranded and linear or covalently closed, circular molecule. Generally, the nucleic acid molecule is present in the form of cDNA. The present invention also comprises other nucleic acid molecules hybridizing to the nucleic acid molecules of the invention or specifically to the sequence shown in SEQ ID NO:1 or a portion or region thereof under conditions of low, preferably medium and most preferably high stringency. A particularly preferred embodiment relates to a nucleic acid molecule comprising the nucleic acid sequence shown in SEQ ID NO:1 or a molecule having a similarity of at least 40%, more preferably at least 45%, still more preferably at least 55% or most preferably at least 65-70% or most preferably a similarity of more than 85% on the level of the nucleic acid sequence or amino acid sequence to at least one or more regions (preferably over the whole region) of the sequence shown in SEQ ID NO:1 and wherein the nucleic acid encodes or is complementary to a sequence encoding an enzyme having FNS II activity.

Furthermore, the present invention comprises nucleic acid molecules in the form of oligonucleotide primers or competent probes for hybridization with a portion of the nucleic acid molecules described above and specifically to that shown in SEQ ID NO:1. The hybridization may be carried out under conditions of low, preferably medium and most preferably high stringency. Preferably, said portion corresponds to the 5' or the 3' end of the gene. For the purposes herein, the 5' end is defined as the region extending mainly between the start codon of the structural gene sequence to the medium region of the gene. The 3' end is considered herein as being the region defining the structural genetic sequence between the medium region of the gene and the stop codon. Therefore, it is obvious that oligonucleotides or probes are able to hybridize to the 5' end or the 3' end or to a region which is common to both the 5' or the 3' end. The present invention comprises all such probes. Preferred oligonucleotides are presented in Example 4.

The nucleic acid or the complementary form thereof may encode the full length enzyme or a portion or derivative thereof. "Derivative" means single or multiple amino acid substitutions, deletions and/or additions with respect to the naturally occurring enzyme while, preferably, the flavone synthase II activity is maintained. In this respect, the nucleic acid according to the present invention comprises the naturally occurring nucleotide sequence encoding FNS II and single or multiple nucleotide substitutions, deletions, and/or additions. The nucleic acid according to the present invention or the complementary form thereof may also encode a portion of FNS II which is either active or inactive. Such a nucleic acid molecule may be used as an oligonucleotide probe, as a primer for polymerase chain reaction, in different mutagenesis techniques, or for the preparation of antisense molecules.

Furthermore, the present invention relates to a recombinant DNA molecule containing a nucleic acid sequence according to the present invention. In a preferred embodiment, the recombinant DNA molecule is a vector or a vector having a promoter. In a particularly preferred embodiment the promoter is capable of expressing the nucleic acid sequence according to the present invention. The nucleic acid molecules of the invention may be present in combination with a vector molecule, e.g. an expression vector, in both orientations. In this respect, the term "vector molecule" is used in its most general meaning to comprise any

intermediary vehicles of the nucleic acid molecule which enable the transfer of the nucleic acid into cells, particularly in plant cells and/or its integration into a genome. Preferably, these vector molecules or portions thereof are used for integration into a plant genome. Such vector molecules may be replicated and/or expressed in prokaryotic cells and/or in eukaryotic cells. An intermediary vehicle may be for example adapted to the use in electroporation, microprojectile bombardement, in the transfer using agrobacteria or in the insertion via DNA or RNA viruses. The intermediary vehicle and/or the nucleic acid molecule contained therein may be stably integrated into the plant genome. Additionally, the nucleic acid molecule may contain also a promoter sequence useful for the initiation of expression of the nucleic acid molecule in a cell, particularly in a plant cell. The nucleic acid molecule and the promoter may also be introduced into the cell using different methods (see above).

The present invention also relates to host cells containing the DNA molecules according to the present invention. The host cells may be prokaryotic or eukaryotic cells, particularly yeast cells, insect cells, plant cells, and mammalian cells.

The present invention further relates to a polypeptide encoded by a nucleic acid according to the invention. In a preferred embodiment the present invention relates to a polypeptide having the amino acid sequence as shown in SEQ ID NO:2 or a portion or derivatives thereof. In another preferred embodiment the polypeptide is derived from a flavone-containing plant, such as e.g. from gerbera (*Gerbera hybrids*), aster (*Callistephus chinensis*), snapdragon (*Antirrhinium majus*), chrysanthemum (*Chrysanthemum indicum*), dahlia (*Dahlia hybrids*), gloxinia (*Sinningia hybrids*), verbena (*Verbena hybrids*), and *Streptocarpus* (*S. hybrids*). In a particularly preferred embodiment the polypeptide of the present invention has flavone synthase II activity.

Derivatives in the sense of the present invention are amino acid insertion derivatives, deletion derivatives and/or substitution amino acid variants of the amino acid sequence of SEQ ID NO:2.

Amino acid insertion derivatives of FNS II according to the present invention comprise both amino and carboxyl fusions as well as insertions of single or

multiple amino acids within the sequence. Insertion amino acid sequence variants are those in which one or more amino acid residues have been introduced into the protein at a predetermined site, although a random insertion together with appropriate screening of the product is also possible. Deletion variants are characterized by the removal of one or more amino acids from the sequence. Substitution amino acid variants are those wherein at least one residue of the sequence has been removed and another residue has been introduced at the same site. Typical substitutions are presented in Table 1.

TABLE 1

Suitable residues for amino acid substitution

Original amino acid	Exemplary substitution by
Aln	Ser
Arg	Lys
Asn	Gln; His
Asp	Glu
Cys	Ser
Gln	Asn
Glu	Asp
Gly	Pro
His	Asn; Gln
Ile	Leu; Val
Leu	Ile; Val
Lys	Arg; Gln; Glu
Met	Leu; Ile
Phe	Met; Leu; Tyr
Ser	Thr
Thr	Ser
Trp	Tyr
Tyr	Trp; Phe
Val	Ile; Leu

In general, amino acids are substituted by amino acids having similar properties such as hydrophobicity, hydrophilicity, electronegativity, very bulky side chains and the like. Amino acid substitutions generally relate to only a single residue whereas insertions typically are directed to a region of 1 to 10 amino acid residues and deletions to a region of 1 to 20 residues. Preferably, deletions and insertions are carried out on adjacent pairs, e.g. a deletion of two residues or an insertion of two residues.

The amino acid variants described above of derivatives according to the present invention may be prepared easily using known techniques for peptide synthesis such as e.g. by solid phase synthesis and similar methods or by recombinant DNA manipulations. Techniques for introducing substitution mutations at predetermined sites in DNA having a known or partially known sequence are well-known and include for example M13 mutagenesis. The manipulation of DNA sequences for the preparation of proteins carrying substitutions, insertions or deletions is detailed for example in Sambrook et al. (1989).

Other examples of recombinant or synthetic mutants and derivatives of FNS II according to the present invention comprise single or multiple substitutions, deletions and/or additions of any molecule associated with the enzyme such as carbohydrates, lipids and/or proteins or polypeptides.

The terms "analogue" and "derivative" also comprise any functional chemical equivalent of FNS II and also any amino acid derivative as already described above.

Another aspect of the present invention relates to recombinant forms of FNS II. Recombinant forms of the enzyme provide the possibility to develop for example more active enzymes or systems for production of various flavones *in vitro* for the use in various fields such as for example in human medicine. The latter system may be for example of use in cancer research.

In another embodiment, the present invention relates to transgenic plants containing a nucleic acid sequence according to the present invention. In a preferred embodiment, the nucleic acid sequence is suitable for expression and optionally can be controlled or is controlled within the plant in conjunction with its development. In another preferred embodiment the transgenic plant is selected from the group of flavone-containing plants such as gerbera (*Gerbera hybrids*), aster (*Callistephus chinensis*), snapdragon (*Antirrhinium majus*), chrysanthemum (*Chrysanthemum indicum*), dahlia (*Dahlia hybrids*), gloxinia (*Sinningia hybrids*), verbena (*Verbena hybrids*), and *Streptocarpus* (*S. hybrids*), contains an

endogenous FNS II and further contains a non-endogenous FNS II nucleic acid sequence according to the present invention.

According to the present invention, a nucleic acid sequence encoding an FNS II or a derivative or portion thereof may be introduced into a plant in one of two possible orientations and may be expressed therein thereby providing the possibility to convert either naringenin (NAR) and/or other suitable substrates, if synthesized within the plant cell, which eventually results in the formation of different flavones. Moreover, the formation of said metabolites may be inhibited by a reduction or elimination of endogenous or existing FNS II activity. In gerbera, the synthesis of flavones results in an alteration of the flower color. Using the flavone-containing, orange colored variety "Th 58" as an example which is heterozygous for the *Fns* locus (*fns*$^{+}$ *fns*) a color variation from dark red (flavone-free; genotype *fns fns*) via different shades of orange-red (flavone-containing; genotype *fns*$^{+}$ *fns*) to a yellow-orange color (very high flavone content; genotype *fns*$^{+}$ *fns*$^{+}$) could be demonstrated in autogamy progeny. This experiment may also be applied to other gerbera varieties heterozygous for the Fns locus (see also Example 2). Expression of the nucleic acid sequence in one of two possible orientations within the plant may be constitutively, inducible or dependent on the development and also tissue-specific. The term "expression" is used in its most general meaning to include the production of RNA or both RNA and protein. It also comprises the partial expression of nucleic acid molecules.

The present invention relates to a method for the preparation of transgenic plants capable of synthesizing FNS II or active mutants or derivatives. Said method comprises the stable transformation of a cell of a suitable plant with a nucleic acid molecule comprising a nucleotide sequence encoding said FNS II under conditions achieving the possible expression of said nucleic acid molecule, regeneration of a transgenic plant from the cell and growth of said transgenic plant for a particular time and under conditions suitable to achieve expression of the nucleic acid. The transgenic plant may exhibit higher values of FNS II activity compared to the value measured in comparable non-transgenic plants, or the values may be lower compared to those of comparable non-transgenic plants.

One aspect of the present invention relates to a method for the preparation of a transgenic plant having a reduced, endogenous or existing FNS II activity. This method comprises the stable transformation of a cell of a suitable plant with a nucleic acid molecule comprising a nucleotide sequence encoding a sequence or a complementary sequence of FNS II, the regeneration of a transgenic plant from the cell, and, if necessary, the raising of this transgenic plant under conditions suitable to achieve expression of nucleic acids.

Another aspect of the present invention relates to a method for the preparation of a genetically engineered plant having a reduced, endogenous or existing FNS II activity. This method comprises the alteration of the FNS II gene by a modification of the endogenous sequence via homologous recombination starting from an appropriately modified gene of an FNS II or a derivative or portion thereof. The gene is introduced into the plant and a genetically engineered plant is regenerated from the cell.

Another aspect of the present invention relates to a method for the preparation of a transgenic flowering plant having altered flower characteristics. This method comprises the introduction of the nucleic acid sequence according to the present invention into a cell of a suitable flavone-free plant, the regeneration of a transgenic plant from the cell, and raising a transgenic plant for a time and under conditions to achieve the expression of the introduced nucleic acid sequence according to the present invention. The transgenic plant may be for example selected from the group of flavone-containing plants consisting of euphorbia (*E. pulcherrima*), cyclamen (*Cyclamen persicum*), rose (*Rosa hybrida*), pelargonium (*P. spec.*), begonia (*B. spec.*), carnation (*Dianthus caryophyllus*), and tulip (*Tulipa hybrids*). In another preferred embodiment, the transgenic plant is capable of expressing an endogenous flavone synthase II. Such a transgenic plant may be for example selected of the group of plants consisting of gerbera (*Gerbera hybrids*), aster (*Callistephus chinensis*), snapdragon (*Antirrhinium majus*), chrysanthemum (*Chrysanthemum indicum*), dahlia (*Dahlia hybrids*), gloxinia (*Sinningia hybrids*), verbena (*Verbena hybrids*), and *Streptocarpus* (*S. hybrids*). In another preferred embodiment this endogenous flavone synthase II is coexpressed during expression of the nucleic acid introduced according to the present invention.

In one embodiment, the endogenously existing flavone synthase II activity is reduced by the introduction of the nucleic acid sequence. This method comprises the stable transformation of a cell of a suitable plant with a nucleic acid sequence according to the invention or a sequence complementary thereto, the regeneration of a transgenic plant from the cell and raising this transgenic plant for a time and under conditions suitable to alter the amount of activity of the endogenous or existing FNS II. Preferably, the altered level is lower than the endogenous or existing level of FNS II activity in a comparable non-transgenic plant. Optionally, for the reduction of the endogenous FNS II activity it is necessary to express the nucleic acid sequence introduced or a complementary sequence thereof. Thus, an expression of the genetic sequence introduced or of its complementary analogue may be required to achieve the desired effect. This substantially means a flowering plant with altered flower characteristics.

In this respect the present invention relates to a method for the preparation of a flowering plant showing different flower characteristics. This method comprises alterations of the FNS II gene by modification of the endogenous sequences via homologous recombination of an appropriately altered gene of FNS II or a derivative or portion thereof, the introduction into the plant cell and the regeneration of the genetically engineered plant from the cell.

Moreover, the nucleic acid molecule according to the present invention may be regulated in a development-depending manner. Generally, an altered inflorescence rules out the possibility of producing a flower having a weaker color or other shades depending on the physiological conditions of the recipient plant. "Recipient plant" refers to a plant which produces a measurable amount of substrate of FNS II enzyme or FNS II itself and has the corresponding physiological properties and the genotype necessary for the development of the desired colors. This includes but is not restricted to the following plants: gloxinia (*Sinningia hybrids*), snapdragon (*Antirrhinium majus*), columnea (*Columnea hybrids*), dahlia (*Dahlia variabilis*), gloxinia (*Sinningia cardinalis*), *Streptocarpus* (*Streptocarpus hybridus*), verbena (*Verbena hybrida*), chrysanthemum (*Chrysanthemum indicum*), peace lily (*Spathiphyllum wallisii*), petunia (*Petunia hybrida*), cyclamen (*Cyclamen persicum*), rose (*Rosa hybrids*), and pelargonium (*P. spec.*).

Accordingly, the present invention relates to a method for the preparation of a transgenic plant expressing, to a measurable extent, a recombinant gene encoding FNS II or a portion thereof or carrying a nucleic acid sequence which is substantially complementary to the full length or a portion of the mRNA molecule which may be easily transcribed, if necessary, to achieve the regulation of FNS II. This method comprises the stable transformation of a cell of a suitable plant with the isolated nucleic acid comprising a nucleotide sequence encoding FNS II or a derivative or a portion thereof or a sequence complementary to the coding nucleotide sequence, if necessary under conditions allowing an expression of said isolated nucleic acid, and the regeneration of a transgenic plant from the cell.

The skilled artisan immediately appreciates the possibilities for use of the present invention such as e.g. for an increase or decrease in the expression of enzymes which occur naturally in a target plant. This will result in different novel flower color shades, for example various orange and dark red shades.

Therefore, the present invention relates to any transgenic plant containing the full length or a portion of the nucleic acid sequence according to the present invention and/or containing a homologous or related form thereof or an antisense form of any of those described, and in particular those transgenic plants exhibiting varying flower characteristics. The transgenic plants may contain nucleic acid molecules introduced therein comprising a nucleotide sequence encoding FNS II or a complementary sequence thereof. Generally, the nucleic acid is stably introduced into the plant genome although the present invention also comprises the introduction of an FNS II nucleic acid sequence within an autonomously replicating nucleic acid sequence such as for example DNA or RNA viruses capable of replicating in a plant cell. Moreover, the present invention also comprises seeds of the transgenic plant, particularly those containing flavones.

Using the FNS II gene according to the present invention, the biosynthesis of flavonoids and the formation of flavones, respectively, in plants may be altered in a targeted manner in different ways.

To begin with, two different approaches must be considered in the field of plants. On the one hand, plants are available which naturally form flavones and the

glycosides thereof, such as *Antirrhinium* and *Verbena* in flowers, *Clerodendron* and *Citrus* in leaves, *Althaea* and *Sophora* (leguminosae) in roots, and *Prunus* and *Pinus spec.* in hardwood (Wollenweber, 1994; Williams and Harborne, 1994). Besides this very large group there are also some plants which lack flavone synthesis in certain tissues. This is the case e.g. in several flowers of important ornamental plants such as *Pelargonium*, *Cyclamen*, and *Petunia* as well as in apple leaves.

Accordingly, the present invention relates to an altered FNS II activity in plants and other organisms which may be achieved both by increasing and by decreasing the naturally occurring FNS II activity by an introduction of the sequence of the present invention. A reduction of the amount of FNS II activity may also be referred to as down regulation.

In flavone-containing plants, the flavone synthesis may be up or down regulated or switched off completely in a targeted manner by means of suitable methods. This has several consequences for the plant. Basically, an alteration of the biosynthetic pathway of this type has an effect on the whole flavone biosynthesis since there is a permanent competition of several enzymes (FNS II, FHT, F3'H, F3',5'H) for the substrate (flavonones, e.g. naringenin). This means in detail that e.g. an up-regulation of FNS II reduces the synthesis of flavonoids in a downstream biosynthetic pathway (Fig. 1A) whereby for example a paler flower color may be achieved. In addition, by this targeted increase in flavone content which may be restricted to specific tissues, the resistance properties and the capability to form symbioses of the plant may be markedly improved. In contrast, a down-regulation results in an increase in the synthesis of other flavonoids which may be more beneficial for the plant. This may affect both the flavonols important for UV protection and the coloring anthocyans or the resistance-inducing proanthocyanidines and phytoalexins. A complete suppression of flavone formation results in an enhancement of the effects of a down-regulation and may remove possible stimulants for insects and thus contribute to the resistance generation.

An entirely new synthesis of flavones may be achieved by the targeted introduction of FNS II into plants lacking a natural activity. Depending on the

substrates present, the flavone content and the flavone pattern may thus be controlled. By establishing this step, flower colors, resistance properties, and the capability to form symbioses with nitrogen-fixing bacteria may be altered in a targeted manner. Moreover, opening a novel biosynthetic pathway may reduce the formation of flavonoids which are less beneficial for the plant (e.g. feed stimulants), and the synthesis of biflavones generated from flavones may be established.

Using these approaches, the flavonoid pattern and content of important flavone-containing or flavone-free agricultural plants may be altered to optimize their positive properties with respect to the biology of men and animals.

In addition, the present invention relates to methods for the targeted alteration of the flavone content and flavone pattern, respectively, in various plant tissues (flowers, roots, leaves etc.) and other organisms, and thus in general it relates to an alteration of the flavonoid composition, particularly the alteration of flower colors by means of copigmentation, of resistance properties, and of the capability of nodulation in the case of leguminous plants.

In another embodiment, the present invention relates to the use of a polypeptide according to the present invention for the synthesis of flavones. In suitable expression systems, the enzyme itself and thereby eventually the natural flavones may be synthesized starting from appropriate substrates. A possibility is the use of suitable expression systems for obtaining health-promoting, natural flavones. These expression systems may be of plant origin or consist of cell cultures or yeast cultures, respectively. The expression of FNS II in plants or in cell cultures may be used to directly obtain the respective flavones wherein the yeast expression system may be preferred for obtaining the intact enzyme. Using this enzyme, chemical or natural precursors (flavanones) may then be reacted to form the corresponding flavone. The flavones synthesized in this manner may for example find use in cancer therapy or contribute to human and animal health in the form of medicaments.

The present invention is explained in detail with respect to the following Figures and Examples. The present invention is illustrated with respect to a nucleic acid

sequence derived form *Gerbera hybrids*. It will now be obvious that similar sequences may be isolated easily from various other sources such as e.g. other plants or specific microorganisms. Examples of other flavone-producing plants include but are not restricted to gloxinia (*Sinningia hybrids*), snapdragon (*Antirrhinium majus*), columnea (*Columnea hybrids*), dahlia (*Dahlia variabilis*), gloxinia (*Sinningia cardinalis*), *Streptocarpus* (*Streptocarpus hybridus*), verbena (*Verbena hybrida*), chrysanthemum (*Chrysanthemum indicum*), peace lily (*Spathiphyllum wallisii*). All these nucleic acid sequences encoding, directly or indirectly, an enzyme of the flavonoid biosynthesis pathway and particularly FNS II are encompassed by the present invention disregarding the source thereof.

FIGURE 1A-C shows a schematic representation of the general flavonoid biosynthesis pathway and the chemical structures of several flavonoids.

The enzymes involved are abbreviated in Figs. 1A and 1B as follows: CHS = chalcone synthase; CHI = chalcone isomerase; FHT = flavanone 3-hydroxylase; DFR = dihydroflavonol 4-reductase; ANS = anthocyanidine synthase; FGT = flavonoid 3-glycosyl transferase; FNS II = flavone synthase II; FLS = flavonol synthase; F3'H = flavonoid 3'-hydroxylase; F3',5'-H = flavonoid 3',5'-hydroxylase. The level of flavone formation is particularly indicated (++++) in Figure 1A. In the upper portion of Figure 1B the FNS II reaction is shown in the presence of NADPH and several common flavanones. In the lower portion, other important flavonoids are shown. Figure 1C describes the flavonoid biosynthesis pathway as present in *Gerbera hybrids*. The following abbreviations have been used for the different flavonoids: THC = tetrahydroxychalcone; PHC = pentahydroxychalcone; NAR = naringenin; ERI = eriodictyol; Ap = apigenin; Lu = luteolin; DHK = dihydrokaempferol; DHQ = dihydroquercetin; Km = kaempferol; Qu = quercetin; LPg = leucopelargonidin; LCy = leucocyanidin; Pg = pelargonidin; Cy = cyanidin.

FIGURE 2 shows the activity or the lack of activity, respectively, of FNS II in enzyme extract from petals of different gerbera lines: "Th 58" (genotype fns^+ fns), "147-150" (fns^+ .), and "147-146" (genotype fns fns). The lines "147-150" and "147-146" are autogamy progeny of "Th 58". The FNS II activity was measured by means of the turnover of ^{14}C-labeled naringenin to the corresponding flavone apigenin.

ABSTRACT

The invention relates to genetic sequences which code for the enzyme of the flavonoid metabolism, in particular, for flavone synthase II (FNS II) or derivates thereof and to their use for specifically modifying the colour of flowers, for modifying the flavone content or expression in leaves, flowers and other plant or organic tissue. The use also covers expression systems for synthesising natural, functional flavones for medical or similar applications, for example, for treating cancer or for improving the human immune defence.

FIGURE 3 shows the FNS II activity (▦) and the accumulation of flavones (□) in line "Th 58" (fns^+ fns) over different developmental stages of the flower. The different flower stages are defined in Example 2. The flavone content was determined by extraction with ethylacetate and HPLC detection as described in Martens and Forkmann (1998).

FIGURE 4A+B (according to Schopfer and Ebel, 1998) shows the known structure of cytochrome P450 sequences containing regions of high sequence conservation. The prolin-rich, the oxygen binding and the heme binding regions are indicated. In Fig. 4B the heme binding region is shown in detail in addition to the primers derived therefrom for DD-RT PCR. A set of eight non-degenerated 5' primers was prepared according to the putative nucleotide sequence.

FIGURE 5 shows a schematic representation of the different cytochrome P450 DNA fragments generated. All clones contain the heme binding site indicated. pDDd7a: a 358 bp fragment could be generated via PCR using the oligonucleotides "Decamer d7" and "Oligo A" with a DNA template recovered from a differentially expressed band.

pTABATA: a 1519 bp fragment was isolated starting from gerbera "Th 58" cDNA via a PCR-supported RACE method using oligonucleotides "GSP7", "GSP8", GSP9", and "AAP" (GIBCO-BRL) or by "backrace", respectively.

pCYPFNS1: a 1589 bp fragment containing an open reading frame was isolated via PCR using oligonucleotides "CypFNS1H" and "CypFNS1R". cDNA of gerbera "Th 58" was used as the template.

FIGURES 6 and 7 are representations of the nucleic acid and the amino acid sequence derived therefrom, respectively, of the full length clone. The **start codon** and the different **stop codons** are indicated separately.

Figure 8 shows a diagram of the restriction sites present for standard restriction enzymes.

FIGURE 9 shows a FNS II assay using yeast microsomes. [^{14}C]-naringenin was used as the substrate. Microsomes were prepared from transformed yeasts (INVSc1 - CypFNS1) and untransformed yeasts (INVSc1). The autoradiograph shows the conversion of [^{14}C]-naringenin to the corresponding flavone, [^{14}C]-apigenin, using an extract of transformed yeast (INVSc1 - CypFNS1). In the control experiment (INVSc1) no activity was measured. The product was identified by co-chromatography with authentic apigenin in four different eluents.

FIGURE 10 shows an autoradiography of an RNA gel blot hybridized with a ^{32}P-labeled cDNA of insert CypFNS1. Each lane contains 20 µg of total RNA which was applied as follows: (1) Simm (genotype fns$^+$.), (2) Delphi (fns$^+$.), (3) T3 (fns fns), (4) 147-150 (fns$^+$.), (5) clivia (fns fns), (6) 147-146 (fns fns), (7) Regina (fns$^+$.), (8) gerbera leaves (fns fns), (9) pool of 10 (fns$^+$.), (10) pool of 10 (fns fns).

EXAMPLE 1

Materials

Chemicals, enzymes and radiochemicals

Naringenin, eriodictyol, apigenin, and luteolin were obtained from Carl Roth (Karlsruhe, Germany). [^{14}C]-naringenin was prepared from [^{14}C]-malonyl CoA (ARC, St. Louis, USA) and p-cumaroyl CoA (Dr. Werner Heller, GSF, Neuherberg, Germany) according to the method described in Britsch et al. (1981) using partially purified chalcone synthase (CHS) and chalcone isomerase from parsley suspension culture. All other enzymes were obtained from commercial suppliers and used according to their specifications.

Bacterial and yeast strains

The following *Escherichia coli* strains were used: TOP10F' and TOP10, both from Invitrogen (Groningen, Netherlands). In addition, the following yeast strain was used: INVSc1 (Invitrogen).

Cloning vectors pCR2.1 and pYES2 were obtained from Invitrogen.

The ligation of insert and vector pCR2.1 and the transformation of bacteria, respectively, was carried out according to the instructions of the manufacturer.

Plant materials

Chemogenetically defined *Gerbera* clonal varieties (Tyrach and Horn, 1997) and autogamy progeny of line "Th 58" were available. In addition, the current cut gerbera varieties "Regina" of Terra Nigra company (DeKwakel, Netherlands) and "Delphi" of Florist company (DeKwakel, Netherlands) were included. A detailed description of the plant material may be found in Table 2.

TABLE 2

Plant material used

Gerbera line	Genotype	Source
Th58	fns^+ fns	Tyrach and Horn, 1997
Delphi	fns^+ fns	Florist, Tyrach und Horn, 1997
Simm	fns^+ fns	Tyrach and Horn, 1997
T3	fns fns	Tyrach and Horn, 1997
147-150	fns^+ fns	Th 58 x S
Clivia	fns fns	Tyrach and Horn, 1997
147-146	fns fns	Th 58 x S
Regina	fns^+ .	Terra Nigra
Regina leaves	fns fns	Terra Nigra
pool of 10 (147-...)	fns^+ .	Th 58 x S
pool of 10 (147-...)	fns fns	Th 58 x S

EXAMPLE 2

Plant cultivation, crossing methods and flower stages

Plants of *Gerbera hybrids* were cultivated in a green house under conditions common in practice. The day length was at least 14 h at a light intensity of 10,000 lux and at 22°C.

Autogamy of the variety "Th 58" (genotype *fns*$^+$ *fns*) was carried out within the same inflorescence or between inflorescences of the same plant. This autogamy experiment may be carried out with any variety or line heterozygous for the *Fns* locus. The corresponding chemogenetic and biochemical methods are detailed in Tyrach and Horn (1997) and Martens and Forkmann (1998). Controlled flowering was achieved by glassine bags put over the flowers. Ideally, the pollinations were repeated up to four times in daily intervals. Further descriptions with respect to the methods of pollination and floral morphology in *Gerbera* may be found in Maurer (1967).

Gerbera flowers were harvested in different developmental stages defined as follows (according to Martens and Forkmann, 1998):
Stage 1: bud closed, petals smaller than 5 mm;
Stage 2: ray flowers visible, 5-10 mm long;
Stage 3: ray flowers 10-15 mm long;
Stage 4: beginning pigmentation, length 15-23 mm;
Stage 5: ligula of ray flowers pigmented, 23-26 mm long;
Stage 6: ray flowers 26-35 mm long;
Stage 7: inflorescence half open, 35-40 mm long;
Stage 8: inflorescence completely opened, 40-50 mm long;
Stage 9: ray flowers 50-55 mm long;
Stage 10: ray flowers 55-60 mm long;
Stage 11: senescent inflorescence, 55-60 mm long.

EXAMPLE 3

Biochemical and enzymological characterization of the autogamy progeny

Known standard methods (Marbry et al., 1970; Harborne, 1967) were used for the extraction and identification of flavonoids. Flavones were additionally detected under UV light (243 nm) prior and after vaporization with ammonia. Flavanones were identified by reduction with sodium borohydride and subsequent treatment with hydrochloric acid vapors (Eigen et al., 1957).

Thin layer chromatography was carried out on precoated cellulose plates G1440 of Schleicher & Schüll company (Dassel, Germany). For this purpose, the following eluents were used: (1) chloroform-acetic acid-water (10:9:1); (2) 30% acetic acid; (3) acetic acid-hydrochloric acid-water (30:3:10); and (4) tert-butanol-acetic acid-water (3:1:1).

The flavone content of buds and flowers during development was determined by extraction of the pigments from petals of the different stages using ethylacetate. The ratio of tissue to extracting agent was 1:40 (g/ml) and the period of extraction was 48 h at 4°C in the dark. Characterization and quantification were performed using HPLC. 10 µl of 75% methanolic extract were applied to and separated on a Spherisorb ODS II column (particle size 5 µm, 250 x 4.6 mm, Bischoff, Leonberg, Germany) (Lange et al., 1994). The detection was done using a diode array detector Model 168 (Beckman, Munich, Germany).

Enzyme preparations and FNS II assays were performed as described in Martens and Forkmann (1998). The preparation was carried out in 6.0 ml Tris-HCl buffer (pH 7.5) containing 28 mmoles/l 2-mercaptoethanol and 10 mmoles/l sodium ascorbate at 4°C with 1.0 g of petals, 0.5 g Dowex (equilibrated in Tris buffer, pH 7.5), and 0.5 g sea sand. After homogenizing in a cooled mortar the homogenate was transferred into Eppendorf tubes and centrifuged twice for 5 min at 10,000 x g. The cleared supernatant was used as raw extract or was used for the precipitation of microsomes with $MgCl_2$ according to Diesperger et al., (1974). The protein content of the preparations was determined according to the method of Bradford (1976).

The standard assay for FNS II contained in a total volume of 200 µl: 175 µl Tris-HCl buffer (pH 7.5), 0.3 nmol radiolabeled substrate (83 Bq; naringenin), 2.0 nmol of unlabeled substrate, 10 µl of 20 mmoles/l NADPH and 15 µl raw extract or microsomal preparation. After an incubation of 20 min at 25°C the reaction was stopped by addition of 20 µl methanol containing a mixture of the corresponding flavonoids. The extraction of the reaction mixture was carried out twice with 100 and 50 µl of ethylacetate. The upper phase was chromatographed on cellulose thin layer plates in eluent 1 (see above). The radioactivity was localized and quantified using a Fuji BAS 1000 Bio-Imaging Analyzer (Fuji Photo Film Co., Tokio, Japan) and the TINA software package (Raytest, Straubenhardt, Germany).

Fig. 2 exemplarily shows the result of the enzyme assays carried out with the *Gerbera* variety "Th 58" and the lines "147-150" and "147-146". The corresponding genotypes are shown in Table 2.

EXAMPLE 4

Oligonucleotide synthesis

The oligonucleotides were synthesized by Metabion company (Martinsried, Germany). The following oligonucleotides were used (5'-3'):

Oligo A :	5'-TTTTTTTTTT T(A,C,G)A-3'	(SEQ ID NO:3)
Oligo C :	5'-TTTTTTTTTT T(A,C,G)C-3'	(SEQ ID NO:4)
Oligo G :	5'-TTTTTTTTTT T(A,C,G)G-3'	(SEQ ID NO:5)
Decamer 1 :	5'-CGCCATTTGG-3'	(SEQ ID NO:6)
Decamer 2 :	5'-CGCCATTCGG-3'	(SEQ ID NO:7)
Decamer 3 :	5'-CGCCCTTTGG-3'	(SEQ ID NO:8)
Decamer 4 :	5'-CGCCCTTCGG-3'	(SEQ ID NO:9)
Decamer 5 :	5'-CGCCGTTTGG-3'	(SEQ ID NO:10)
Decamer 6 :	5'-CGCCGTTCGG-3'	(SEQ ID NO:11)
Decamer 7 :	5'-CGCCTTTTGG-3'	(SEQ ID NO:12)
Decamer 8 :	5'-CGCCTTTCGG-3'	(SEQ ID NO:13)
GSP7 :	5'-ATCTTCAAAGTGTTTCCTCGTTCC-3'	(SEQ ID NO:14)
GSP8 :	5'-AATGGAACACACACAAAATCTACC-3'	(SEQ ID NO:15)
GSP9 :	5'-TCACCACTGAGAGTTCTCTCATGG-3'	(SEQ ID NO:16)
AAP :	5'-GGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG-3'	(SEQ ID NO:17)
Backrace :	5'-GCCACGCGTCGACTAGTACG-3'	(SEQ ID NO:18)
CypFNS1H :	5'-CAAAGGATCCCAACACCATGAATACACTCC-3'	(SEQ ID NO:19)
CypFNS1R :	5'-AGATAGACCGACTGCCATCAAGAAAGC-3'	(SEQ ID NO:20)

The three oligomers and eight decamers were synthesized according to Schopfer and Ebel (1998).

EXAMPLE 5

Cloning of a P450 fragment from *Gerbera hybrids*

Isolation of total RNA form *Gerbera* petals

Total RNA from *Gerbera* petals was isolated according to a method described in Guiliano et al. (1993) from various defined genotypes (*fns*+ . or *fns fns*; Tab. 2) of stages 2-4 in which the FNS II activity increases (Fig. 3). 1.2 g of plant material frozen in liquid nitrogen was ground to a fine powder in a cooled mortar and transferred into a cooled Corex tube. The tissue was homogenized by thorough vortexing in 3 ml of charged extraction buffer consisting of 4 M guanidinium thiocyanate, 0.15 M sodium acetate (pH 5.3), 0.2% sodium sarcosinate and 0.7%

ß-mercaptoethanol and 2.4 ml water-equilibrated phenol (saturated with 0.1 M citrate buffer, pH 4.3, Sigma, Deisenhofen, Germany). After addition of 0.6 ml chloroform the thoroughly mixed homogenate was kept on ice for 20 min and then centrifuged at 15,000 x g (Sorvall RC-5B plus; SS34). The removed upper phase was added with 1 vol. of isopropanol and incubated for another 60 min on ice. After centrifugation for 30 min at 15,000 x g (Sorvall, see above) the upper phase was discarded and the pellet resuspended in sterile H_2O. To remove the polysaccharides, the solution was added with 100% ethanol (20% (v/v) final concentration), incubated for 20 min on ice, and centrifuged 10 min at 10,000 x g and 4°C. The supernatant containing the nucleic acids was added with 1/3 vol. of 8 M lithium chloride. After incubation for 30 min on ice centrifugation for 20 min at 15,000 x g was carried out and the supernatant discarded. The precipitated RNA was washed twice each with 1 ml 80% ethanol and then resuspended in 50 µl H_2O and stored at -70°C. Determination of the RNA concentration was carried out spectrophotometrically at a wave length of 260 nm (Pharmacia Biochrome 4060). If necessary, poly $(A)^+$ RNA was additionally isolated from the total RNA by two cycles of oligo (dT) cellulose chromatography (Sambrook et al., 1989).

Reverse transcription of RNA

5 µg of total RNA or 500 ng of poly $(A)^+$ RNA isolated from the different genotypes or flower stages, respectively, as described above were transcribed into cDNA in a 25 µl sample using one of the three oligo (dT) primers each by reverse transcriptase (SuperScript™ II, GIBCO BRL, Paisley, Great Britain). The RNA was added with 1 µl of the respective oligo (dT) primer (1 µM final) and water up to a volume of 17 µl. The sample was then denatured at 70°C for 10 min and afterwards placed on ice. After addition of 4 µl 5 x SuperScript first strand buffer, 2 µl 100 mM dithiothreitol, 1µl 10 mM dNTP mix, the sample was preincubated for 2 min at 42°C. Afterwards, 1 µl (200 U) of SuperScript™ reverse transcriptase was pipetted directly into the sample. The reaction was incubated for 60 min at 42°C and then for 15 min at 70°C. This provided the following cDNAs: *fns*$^+$ A, C or G and corespondingly for the recessive line *fns*$^-$ A, C or G. These cDNAs were used directly as templates for the PCR sample.

PCR amplification using p450-specific primers

The amplification of different cDNAs was performed by means of PCR wherein besides the oligo (dT) anchor primer a second non-degenerated P450-specific primer (Decamer 1-8) was used. The PCR sample contained the following components in a total volume of 20 µl: 6.2 µl water, 1 µl 20 x polymerase buffer, 2.5 mM $MgCl_2$, 0.2 µM dNTP mix, 1 µl ^{35}S-ATP [1000 Ci/mmol] (ICN Pharmaceuticals, Irvine, USA), 0.5 µM of one of eight decamer primers, 1 µM of oligo (dT) primer specific for the cDNA, 4 µl of the cDNA sample described above, and 0.2 µl 5 U/µl Replitherm polymerase (Epicentre, Madison, USA). The following PCR parameters were used: denaturation step at 94°C for 10 min, then 40 cycles at 94°C for 30 seconds, 40°C (annealing) for 2 min, and 72°C (extension) for 30 sequence, followed by a final extension at 72°C for 7 min. 4 µl of the PCR product were then added with 2 µl formamide loading buffer (80% formamide; 10 mM EDTA (pH 8.0), 1 mg/ml xylencyanol FF and 1 mg/ml bromophenol Blue, mixed, denatured at 95°C for 2 min, and then placed directly on ice. The sample prepared in this manner were loaded to a 5% denaturing polyacrylamide gel and separated for 3 hours at a maximum of 40 W. Subsequently, the gel was transferred to Whatman paper and fixed in a gel dryer. The radioactivity or differential bands, respectively, were localized using a Fuji BAS 1000 Bio-Imaging Analyzer (Fuji) and the TINA software package (Raytest). Differentially expressed bands at a size of 300 to 500 bp were cut together with the Whatman paper from the fixed gel using a sharp blade, transferred to an Eppendorf tube and rehydrated in 100 µl water for 10 min at room temperature. Subsequently, the tube was incubated at 100°C for 10 min and centrifuged for 2 min at full speed in a table centrifuge to remove residual gel and paper. The supernatant was transferred into a new Eppendorf tube. After addition of 10 µl 3 M sodium acetate, 5 µl 10 mg/ml glycogen as a carrier and 400 µl 100% ethanol the DNA was precipitated for 60 min at -70°C. The precipitated DNA was then pelleted by centrifugation at 4°C and 14,000 rpm, washed twice with 85% ice-cold ethanol, dried in air and resuspended in 10µl water. The reamplification of the cDNA obtained in this manner was carried out in PCR samples of 50 µl containing the following components: 27.8 µl water, 2 µl 20 x polymerase buffer, 4 µl 25 mM $MgCl_2$, 3.2 µl 500 µM dNTP mix, 4 µl of 5µM of the corresponding decamer primer, 4 µl 25 µM of the corresponding oligo (dT) primer, 4 µl of the eluted DNA described above and 0.5 µl 5U/µl Replitherm polymerase (Epicentre). The PCR parameters were the same as in the first amplification. The PCR

products were separated using a 1.5% agarose gel and the corresponding amplificates were cloned into vector TOPO pCR2.1 and then transformed into TOPO 10F' one-shot competent cells (Invitrogen) according to the instructions of the manufacturer. Plasmid isolation from bacteria identified by means of blue-white screening was performed using the Plasmid Miniprep Quantum Prep kit (Bio-Rad, Munich, Germany) according to the instructions of the manufacturer. The inserts identified after digestion with the appropriate restriction enzymes (e.g. Eco RI; Boehringer, Mannheim, Germany) and separation on a 1.5% agarose gel having a length between 300 and 500 bp were eluted from the gel using the QUIAEX II gel elution kit (QUIAGEN, Hilden, Germany). These DNA fragments were labeled with ^{32}P using the Rediprime Labelling kit (Amersham, Braunschweig, Germany) and used for Northern blot analyses. DNA sequencing of these and other clone was carried out substantially according to the method described by Sanger et al. (1977) using Sequenase enzyme Version 2.1 (Amersham, Braunschweig, Germany).

EXAMPLE 6

Northern blot analysis

Total RNA was isolated as described in Example 5. 10 µg of different total RNA samples were electrophoresed on a 2.2 M formaldehyde/1.2% (w/v) agarose gel. The running buffer contained 20 mM MOPS (pH 7.0), 5 mM sodium acetate and 1 mM EDTA (pH 7.0). The RNA was transferred to Hybond-NX membrane (Amersham) according to the instructions of the manufacturer and hybridized with a ^{32}P-labeled Eco RI-Eco RI pDDd7a cDNA fragment. Prehybridization (1 to 3 hours at 42°C) and hybridization (16 to 24 hours at 42°C) were performed in 50% deionized formamide, 5 x SSPE, 5 X Denhardt's, 0.5% SDS. Denatured herring sperm DNA (100 µg/ml) was added in the hybridization step together with the ^{32}P-labeled probe. The filters were washed twice for 15 min at 42°C in 2 x SSPE, 1% SDS (w/v) and then once or twice at 65°C with 1 x SSPE, 1% SDS (w/v). The radioactivity was localized and quantified using a Fuji BAS 1000 Bio-Imaging Analyzer (Fuji) and the TINA software package (Raytest).

Northern blot analyses revealed that the gene corresponding to cDNA clone pDDd7a is only expressed in lines carrying the fns^{+} . genotype. In lines having the recessive genotype fns fns no hybridization signal could be detected (Fig. 10). Moreover, the expression pattern over the different flower stages is parallel to the

enzyme activity and the flavone accumulation, respectively, measured in the flowers (Martens and Forkmann, 1998).

EXAMPLE 7

Isolation of a full-length clone of pDDd7a

cDNA clone pDDd7a is no full length clone but covers only the region from the heme binding site to the poly (A^+) end (3' end) of the sequence including several stop codons. To obtain the full length clone of pDDd7a a PCR-supported RACE method according to Frohmann et al. (1988) using the 5' RACE system version 2.0 (GibcoBRL) was used.

First, several gene-specific 5'-RACE primers (GSP7-9) on the basis of pDDd7a and in addition the nested amplification primer "backrace" were constructed. Using GSP7, the total RNA was transcribed into cDNA according to the method described in Example 5. Subsequently, the first strand product was purified from the excess of nucleotides and GSP7 using the High Pure PCR Product Purification kit (Boehringer Mannheim) according to the instructions of the manufacturer. To the purified cDNA an oligo-dC tail was added using terminal transferase (TdT). The tailing sample was as follows: 6.5 µl water, 5.0 µl 5 x tailing buffer, 2.5 µl 2 mM dCTP and 10 µl cDNA. This mix was denatured for 3 min at 94°C and then placed on ice for 1 min. The reaction was started by addition of 1 µl TdT and then incubated for 10 min at 37°C. Inactivation of the enzyme was carried out by incubation for 10 min at 65°C. PCR amplification of the dC tailed cDNA was performed in 0.5 ml thin-walled PCR tubes according to the following protocol: single denaturation for 2 min at 94°C, 35 subsequent cycles consisting of 1 min at 94°C, 57°C for 1 min, and 72°C for 2 min. A final extension step of 7 min was also performed. The PCR sample contained the following components: 31.5 µl water, 5.0 µl 10 x PCR buffer, 3.0 µl 25 mM $MgCl_2$, 1.0 µl 10 mM dNTPs, 2.0 µl 10 µM GSP8, 2.0 µl 10 µM AAP, 5.0 µl dC tailed cDNA and 0.5 µl 5 U/µl Taq DNA polymerase (Promega, Madison, USA). 15 µl of 5'-RACE product were analyzed on a 1.5% agarose gel using an appropriate length marker. Specific single bands in a region of 1.5 kb were cloned and verified by Northern blot and sequence analysis, respectively.

Using the 5'-RACE a specific 1.5 kb fragment (pTABATA) was amplified which hybridizes only with total RNA of gerbera lines having the fns^+ . genotype and not with RNA from recessive genotypes. Within the region of the gene-specific primer

(GSP8) up to the heme binding site, this novel fragment is homologous to fragment pDDd7a. A full length clone (1698 bp) contains an open reading frame and shows a homology of 58% on the amino acid level to cytochrome P450 clone CYP93B1 (Akashi et al., 1998).

EXAMPLE 8

Expression of pCYPFNS1 in yeast

Construction of pYeCYPFNS1

A 1.5 kb Bam HI-Eco RI fragment corresponding to pCYPFNS1 was ligated into Bam HI-Eco RI opened yeast expression vector pYES2. The resulting plasmid referred to as pYeCYPFNS1 contained the pCYPFNS1 cDNA fragment in the sense orientation as the insert.

Yeast transformation

The yeast strain INVSc1 was transformed with plasmid pYeCYPFNS1 following the protocol according to Gietz et al. (1992). The selection of transformed yeast cells was carried out via a complementation marker.

Preparation of yeast microsomes for flavone synthase II assays

Individual colonies of INVSc1/pYeCYPFNS1 and INVSc1 grown on selection medium SGI (20 g glucose (w/v), 1 g peptone (Fluka), 6.7 g yeast nitrogen base without amino acids (Difco) and 20 mg L-tryptophane (Fluka) per liter) were then inoculated in 5 x 5 ml SGI broth and incubated at 200 rpm and 30°C for 24 hours. At an OD_{600} of 0.2-0.4 of a 1:10 dilution of the preculture it was completely inoculated into 250 ml YPGE (5 g glucose, 10 g peptone, 10 g yeast extract (Fluka) and 3 vol.% ethanol per liter). The main culture was incubated at 30°C and 120 rpm. At an OD_{600} of 0.8 to 1.2 the induction was carried out by addition of 27 ml 200 g/l sterile galactose solution.

After 12 to 15 hours at an OD_{600} of 0.6 to 1.2 of a 1:10 dilution of the main culture the yeast cells were harvested by centrifugation, washed once with TEK (50 mM Tris-HCl, pH 7.4, 1 mM EDTA, and 0.1 M KCl) and resuspended in TES-B* (50 mM Tris-HCl pH 7.4, 1 mM EDTA, 0.6 mM sorbitol, and 2 mM DTT). Disruption of the yeast cells was performed at 4°C using 15 g glass beads (Sigma) per sample. The glass beads were then washed three times with 5 ml TES-B* and the combined supernatant was adjusted with 4 M NaCl to a final concentration of 0.15 M. The microsomes were precipitated by addition of 2.5 g PEG-4000 (Fluka)

and following a washing step with 2 ml TES-B* were homogenized in 2.5 ml TEG* (50 mM Tris-HCl pH 7.4, 1 mM EDTA, 2 mM DTT) in a Potter. The resulting homogenate served as microsomal enzyme source for FNS II assays.

The FNS II activity was measured according to the method of Martens and Forkmann (1998). The standard assay for flavone synthase II contained in a total volume of 200 µl: 140 µl Tris-HCl buffer (pH 7.5), 0.3 nmoles of radiolabeled substrate (83 Bq; [^{14}C]-naringenin), 10 µl 20 mmoles/l NADPH and 50 µl of the yeast microsomal preparation. After an incubation for 20 min at 25°C the reaction was stopped by addition of 20 µl methanol containing a mixture of naringenin and apigenin (product). Extraction of the reaction mixture was carried out twice with 100 or 50 µl ethylacetate, respectively. The upper phase was chromatographed on cellulose thin layer plates in eluents 1 to 4 (see above). The radioactivity was localized and quantified using a Fuji BAS 1000 Bio-Imaging Analyzer (Fuji) and the TINA software package (Raytest).

The enzyme extract prepared from INVSc1/pYeCYPFNS1 showed a clear FNS II activity whereas the corresponding fraction from untransformed yeasts showed no activity (Fig. 9). The results of the yeast expression confirm that the cDNA insert pCYPFNS1 encodes an FNS II enzyme. Moreover, the result reveals that expression of the enzyme encoded by the *Gerbera* cDNA clone is sufficient in yeast to achieve a direct formation of flavones. This indicates that only a single enzyme is required for introducing the double bond between C2 and C3 (Fig. 1B) and that the cDNA clone described by Akashi et al., (1998) does not represent FNS II but rather a flavanone 2-hydroxylase.

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SEQUENCE LISTING

GENERAL INFORMATION:

APPLICANT:

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TOWN: Tiefenbach
COUNTRY: Germany
POSTAL CODE: 84184

NAME OF THE INVENTION:

Genetic sequence which codes for the flavone synthase II enzyme and use of the same

NUMBER OF SEQUENCES: 20

COMPUTER READABLE FORM:

DATA MEDIUM: Diskette
COMPUTER: IBM PC-compatible
OPERATING SYSTEM: Windows 3.11
SOFTWARE: Microsoft Word 6.0

INFORMATION FOR SEQ ID-NO:1:

SEQUENCE CHARACTERISTICS:

LENGTH: 1697 base pairs
TYPE: nucleic acid
STRANDEDNESS: double
TOPOLOGY: linear

MOLECULE TYPE: DNA

SEQUENCE DESCRIPTION: SEQ ID-NO:1:

ATGTCCTAAC ACAACCCAAC ACCATGAATA CACTCCAACT CATCTTCCTC CTCTTCTTCT 60
TCCCAACCTT ACTCTTCCTC TACTGTCTCC CCTACAAAAG AAACCAAAAC CACCGCCGTC 120
TTCCGCCGTC CCCGCCATCT TTTCCGATCA TCGGCCACCT CCACCATCTC GGCCCACTCA 180
TCCACCAATC CTTCCACGCT CTCTCCACTC GCTACGGCTC TCTAATCCAC CTCCGTCTCG 240
GCTCAGTCCC ATGCGTCGTC GTTTCAACCC CAGACCTCGC CAAAGACTTC CTCAAAACAA 300
ACGAACTCGC GTTCTCATCA AGAAAACACT CCTTAGCCAT CGACCACATC ACCTATGGCG 360

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TGGAGCTTTT GGGCACCCAG AATCTCAGCC ATTTCCTACC CATTCGAACC CATGAAATTC 480
GCGAGCTTCT TCGAACGTTA ATGGTGAAAT CTAGGGCAAA GGAGAGAGTA AACTTGACGG 540
AAGAGTTGTT GAAGTTGACC AACAATGTGA TAAGTCAAAT GATGATGAGC ATTAGGTGTT 600
CGGGGACGAA TAGTGAGGCT GATGAAGCAA AGAATCTTGT TCGGGAAGTG ACCAAAATTT 660
TTGGACAGTT TAATGTTTCA GATTTCATAT GGTTTTGTAA GAACATAGAT TTGCAAGGGT 720
TTAAGAAGAG GTACGAGGGT ACACATAGAA GATATGATGC TTTGCTTGAA AGGATTATAA 780
TGGGGAGGGA AGAAAATAGA AGAAGAGGGA AGATAAAAGA TGGTGAAGGG AAAGATTTTC 840
TTGATATGTT ACTTGATGTT TTGGAGGATG GTAAGGCAGA GATTAAAATT ACTAGAGACC 900
ACATCAAAGC CTTGATTTTG GACTTTCTTA CAGCTGGGAC GGATACCACC GCGATTGCAA 960
TTGAATGGGC ACTAGTCGAA TTGATAAACA ACCCGAACGC TCTCGAGAAA GCAAGACAAG 1020
AGATTGATCA GGTCATCGGT GATGAGAGGC TAGTTCAAGA ATCAGACACG CCTAACCTCC 1080
CTTATATCCA AGCTATCATA AAGGAAGCCC TACGACTTCA CCCACCAATC CCAATGTTGA 1140
TTCGCAAGTC AACAGAAAAT GTAATTGTTC AGGGGTATGA CATCCCAGCC GGCACCTTGT 1200
TGTTTGTCAA TATTTGGTCC ATTGGAAGAA ACCCTCAATG TTGGGAAACC CCTTTAGAGT 1260
TCAAGCCTCA TCGGTTTTTG GATGGTGGTG ACCTTAAAAG CTCTTTAGAT ATTAAAGGCC 1320
ACAATTTTCA ACTATTGCCT TTTGGGACGG GGAGGAGAGG GTGTCCTGGT GTTAATTTGG 1380
CCATGAGAGA ACTCTCAGTG GTGATTGCAA ACCTCATACA ATGCTTTGAT TGGGATGTTG 1440
TAGGTGAACG ACTATTGAAT ACAGATGAAC GTGCTGGATT GACGGCTCCA AGGGCGGTAG 1500
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TTTATTTGTT GTTGCTTTCT TGATGGCAGT CGGTCTATCT ATAGGTCATA ATACCTTGGG 1620
ACTCACGTGT TTGAATCTTA ATACGCTTTT AGTACATTGC TTATCGTATA TCTTGGGTAT 1680
GCATGAAAAA AAAAAAA

INFORMATION FOR SEQ ID-NO:2:

SEQUENCE CHARACTERISTICS:
LENGTH: 511 amino acids
TYPE: amino acid
TOPOLOGy: linear

MOLECULE TYPE: protein

SEQUENCE DESCRIPTION: SEQ ID-NO:2:

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5 10
15
Leu Phe Leu Tyr Cys Leu Pro Tyr Lys Arg Asn Gln Asn His Arg Arg
20 25 30

Leu Pro Pro Ser Pro Pro Ser Phe Pro Ile Ile Gly His Leu His His
35 40 45
Leu Gly Pro Leu Ile His Gln Ser Phe His Ala Leu Ser Thr Arg Tyr
50 55 60
Gly Ser Leu Ile His Leu Arg Leu Gly Ser Val Pro Cys Val Val Val
65 70 75
80

Ser Thr Pro Asp Leu Ala Lys Asp Phe Leu Lys Thr Asn Glu Leu
Ala

85 90 95

Phe Ser Ser Arg Lys His Ser Leu Ala Ile Asp His Ile Thr Tyr
Gly

100 105 110

Val Ala Phe Ala Phe Ala Pro Tyr Gly Thr Tyr Trp Lys Phe Ile
Lys

115 120 125

Lys Leu Phe Thr Val Glu Leu Leu Gly Thr Gln Asn Leu Ser His
Phe

130 135 140

Leu Pro Ile Arg Thr His Glu Ile Arg Glu Leu Leu Arg Thr Leu
Met

145 150 155

160

Val Lys Ser Arg Ala Lys Glu Arg Val Asn Leu Thr Glu Glu Leu
Leu

165 170 175

Lys Leu Thr Asn Asn Val Ile Ser Gln Met Met Met Ser Ile Arg
Cys

180 185 190

Ser Gly Thr Asn Ser Glu Ala Asp Glu Ala Lys Asn Leu Val Arg
Glu

195 200 205

Val Thr Lys Ile Phe Gly Gln Phe Asn Val Ser Asp Phe Ile Trp
Phe

210 215 220

Cys Lys Asn Ile Asp Leu Gln Gly Phe Lys Lys Arg Tyr Glu Gly
Thr

225 230 235

240

His Arg Arg Tyr Asp Ala Leu Leu Glu Arg Ile Ile Met Gly Arg

Glu

245 250 255

Glu Asn Arg Arg Arg Gly Lys Ile Lys Asp Gly Glu Gly Lys Asp

Phe

260 265 270

Leu Asp Met Leu Leu Asp Val Leu Glu Asp Gly Lys Ala Glu Ile

Lys

275 280 285

Ile Thr Arg Asp His Ile Lys Ala Leu Ile Leu Asp Phe Leu Thr

Ala

290 295 300

Gly Thr Asp Thr Thr Ala Ile Ala Ile Glu Trp Ala Leu Val Glu

Leu

305 310 315

320

Ile Asn Asn Pro Asn Ala Leu Glu Lys Ala Arg Gln Glu Ile Asp

Gln

325 330 335

Val Ile Gly Asp Glu Arg Leu Val Gln Glu Ser Asp Thr Pro Asn

Leu

340 345 350

Pro Tyr Ile Gln Ala Ile Ile Lys Glu Ala Leu Arg Leu His Pro

Pro

355 360 365

Ile Pro Met Leu Ile Arg Lys Ser Thr Glu Asn Val Ile Val Gln

Gly

370 375 380

Tyr Asp Ile Pro Ala Gly Thr Leu Leu Phe Val Asn Ile Trp Ser Ile

385 390 395 400

Gly Arg Asn Pro Gln Cys Trp Glu Thr Pro Leu Glu Phe Lys Pro His

405 410 415

Arg Phe Leu Asp Gly Gly Asp Leu Lys Ser Ser Leu Asp Ile Lys Gly

420 425 430

His Asn Phe Gln Leu Leu Pro Phe Gly Thr Gly Arg Arg Gly Cys Pro

435 440 445

Gly Val Asn Leu Ala Met Arg Glu Leu Ser Val Val Ile Ala Asn Leu

450 455 460

Ile Gln Cys Phe Asp Trp Asp Val Val Gly Glu Arg Leu Leu Asn Thr

465 470 475 480

Asp Glu Arg Ala Gly Leu Thr Ala Pro Arg Ala Val Asp Phe Val Cys

485 490 495

Val Pro Leu Glu Arg Gly Asn Thr Leu Lys Ile Leu Gly Ser Asn

500 505 510

INFORMATION FOR SEQ ID-NO:3:

SEQUENCE CHARACTERISTICS:
LENGTH: 12 base pairs

TYPE: nucleic acid
STRANDEDNESS: single
TOPOLOGY: linear

MOLECULE TYPE: synthetic oligonucleotide

SEQUENCE DESCRIPTION: SEQ ID-NO:3:

TTTTTTTTTT NA
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INFORMATION FOR SEQ ID-NO:4:

SEQUENCE CHARACTERISTICS:
LENGTH: 12 base pairs
TYPE: nucleic acid
STRANDEDNESS: single
TOPOLOGY: linear

MOLECULE TYPE: synthetic oligonucleotide

SEQUENCE DESCRIPTION: SEQ ID-NO:4:

TTTTTTTTTT NC
12

INFORMATION FOR SEQ ID-NO:5:

SEQUENCE CHARACTERISTICS:
LENGTH: 12 base pairs
TYPE: nucleic acid
STRANDEDNESS: single
TOPOLOGY: linear

MOLECULE TYPE: synthetic oligonucleotide

SEQUENCE DESCRIPTION: SEQ ID-NO:5:

TTTTTTTTTT NG
12

INFORMATION FOR SEQ ID-NO:6:

SEQUENCE CHARACTERISTICS:
LENGTH: 10 base pairs
TYPE: nucleic acid
STRANDEDNESS: single
TOPOLOGY: linear

MOLECULE TYPE: synthetic oligonucleotide

SEQUENCE DESCRIPTION: SEQ ID-NO:6:

CGCCATTTGG
10

INFORMATION FOR SEQ ID-NO:7:

SEQUENCE CHARACTERISTICS:
LENGTH: 10 base pairs
TYPE: nucleic acid
STRANDEDNESS: single
TOPOLOGY: linear

MOLECULE TYPE: synthetic oligonucleotide

SEQUENCE DESCRIPTION: SEQ ID-NO:7:

CGCCATTCGG
10

INFORMATION FOR SEQ ID-NO:8:

SEQUENCE CHARACTERISTICS:
LENGTH: 10 base pairs
TYPE: nucleic acid
STRANDEDNESS: single
TOPOLOGY: linear

MOLECULE TYPE: synthetic oligonucleotide

SEQUENCE DESCRIPTION: SEQ ID-NO:8:

CGCCCTTTGG
10

INFORMATION FOR SEQ ID-NO:9:

SEQUENCE CHARACTERISTICS:
LENGTH: 10 base pairs
TYPE: nucleic acid
STRANDEDNESS: single
TOPOLOGY: linear

MOLECULE TYPE: synthetic oligonucleotide

SEQUENCE DESCRIPTION: SEQ ID-NO:9:

CGCCCTTCGG
10

INFORMATION FOR SEQ ID-NO:10:

SEQUENCE CHARACTERISTICS:
LENGTH: 10 base pairs
TYPE: nucleic acid
STRANDEDNESS: single
TOPOLOGY: linear

MOLECULE TYPE: synthetic oligonucleotide

SEQUENCE DESCRIPTION: SEQ ID-NO:10:

CGCCGTTTGG
10

INFORMATION FOR SEQ ID-NO:11:

SEQUENCE CHARACTERISTICS:
LENGTH: 10 base pairs
TYPE: nucleic acid
STRANDEDNESS: single
TOPOLOGY: linear

MOLECULE TYPE: synthetic oligonucleotide

SEQUENCE DESCRIPTION: SEQ ID-NO:11:

CGCCGTTCGG
10

INFORMATION FOR SEQ ID-NO:12:

SEQUENCE CHARACTERISTICS:
LENGTH: 10 base pairs
TYPE: nucleic acid
STRANDEDNESS: single
TOPOLOGY: linear

MOLECULE TYPE: synthetic oligonucleotide

SEQUENCE DESCRIPTION: SEQ ID-NO:12:

CGCCTTTTGG
10

INFORMATION FOR SEQ ID-NO:13:

SEQUENCE CHARACTERISTICS:
LENGTH: 10 base pairs
TYPE: nucleic acid
STRANDEDNESS: single
TOPOLOGY: linear

MOLECULE TYPE: synthetic oligonucleotide

SEQUENCE DESCRIPTION: SEQ ID-NO:13:

CGCCTTTCGG
10

INFORMATION FOR SEQ ID-NO:14:

SEQUENCE CHARACTERISTICS:
LENGTH: 24 base pairs
TYPE: nucleic acid
STRANDEDNESS: single
TOPOLOGY: linear

MOLECULE TYPE: synthetic oligonucleotide

SEQUENCE DESCRIPTION: SEQ ID-NO:14:

ATCTTCAAAG TGTTTCCTCG TTCC
24

INFORMATION FOR SEQ ID-NO:15:

SEQUENCE CHARACTERISTICS:
LENGTH: 24 base pairs
TYPE: nucleic acid
STRANDEDNESS: single
TOPOLOGY: linear

MOLECULE TYPE: synthetic oligonucleotide

SEQUENCE DESCRIPTION: SEQ ID-NO:15:

AATGGAACAC ACACAAAATC TACC
24

INFORMATION FOR SEQ ID-NO:16:

SEQUENCE CHARACTERISTICS:
LENGTH: 24 base pairs
TYPE: nucleic acid
STRANDEDNESS: single
TOPOLOGY: linear

MOLECULE TYPE: synthetic oligonucleotide
SEQUENCE DESCRIPTION: SEQ ID-NO:16:

TCACCACTGA GAGTTCTCTC ATGG
24

INFORMATION FOR SEQ ID-NO:17:

SEQUENCE CHARACTERISTICS:
LENGTH: 36 base pairs
TYPE: nucleic acid
STRANDEDNESS: single
TOPOLOGY: linear

MOLECULE TYPE: synthetic oligonucleotide

SEQUENCE DESCRIPTION: SEQ ID-NO:17:

GGCCACGCGT CGACTAGTAC GGGNNGGGNN GGGNNG
36

CHARACTERISTIC:
NAME / KEY: other characteristics
LOCATION:24
OTHER INFORMATION: N is i

CHARACTERISTIC:
NAME / KEY: other characteristics
LOCATION:25
OTHER INFORMATION: N is i

CHARACTERISTIC:
NAME / KEY: other characteristics
LOCATION:29
OTHER INFORMATION: N is i

MERKMAL:
CHARACTERISTIC:
NAME / KEY: other characteristics
LOCATION:30
OTHER INFORMATION: N is i

CHARACTERISTIC:
NAME / KEY: other characteristics
LOCATION:34
OTHER INFORMATION: N is i

CHARACTERISTIC:
NAME / KEY: other characteristics
LOCATION:35
OTHER INFORMATION: N is i

INFORMATION FOR SEQ ID-NO:18:

SEQUENCE CHARACTERISTICS:
LENGTH: 20 base pairs
TYPE: nucleic acid
STRANDEDNESS: single
TOPOLOGY: linear

MOLECULE TYPE: synthetic oligonucleotide

SEQUENCE DESCRIPTION: SEQ ID-NO:18:

GCCACGCGTC GACTAGTACG
20

INFORMATION FOR SEQ ID-NO:19:

SEQUENCE CHARACTERISTICS:
LENGTH: 30 base pairs
TYPE: nucleic acid
STRANDEDNESS: single
TOPOLOGY: linear

MOLECULE TYPE: synthetic oligonucleotide

SEQUENCE DESCRIPTION: SEQ ID-NO:19:

CAAAGGATCC CAACACCATG AATACACTCC
30

INFORMATION FOR SEQ ID-NO:20:

SEQUENCE CHARACTERISTICS:
LENGTH: 27 base pairs
TYPE: nucleic acid
STRANDEDNESS: single
TOPOLOGY: linear

MOLECULE TYPE: synthetic oligonucleotide

SEQUENCE DESCRIPTION: SEQ ID-NO:20:

AGATAGACCG ACTGCCATCA AGAAAGC
27

English translation of amended claims
1 to 25 as filed in response to the
Written Opinion dated July 2, 2001

CLAIMS

1. A nucleic acid sequence encoding a flavone synthase II (FNS II) selected from the group consisting of:

(a) the nucleic acid sequence of SEQ ID NO:1 or a portion thereof encoding a protein or a polypeptide having the biological activity of a flavone synthase II,

(b) a nucleic acid sequence hybridizing under highly stringent conditions to the nucleic acid sequence of (a) and/or having a homology of at least 40% to this sequence and encoding a protein or polypeptide having the biological activity of flavone synthase II,

(c) a nucleic acid sequence which is degenerated with respect to a nucleic acid sequence according to (a) or (b).

2. A nucleic acid sequence which is complementary to the nucleic acid sequence according to claim 1.

3. A nucleic acid sequence according to claim 1 or claim 2 wherein the nucleic acid sequence is DNA or RNA.

4. A nucleic acid sequence according to claim 1 wherein the nucleic acid sequence is derived from a plant from the group of plants consisting of gerbera (*Gerbera hybrids*), aster (*Callistephus chinensis*), snapdragon (*Antirrhinium majus*), chrysanthemum (*Chrysanthemum indicum*), dahlia (*Dahlia hybrids*), gloxinia (*Sinningia hybrids*), verbena (*Verbena hybrids*), and *Streptocarpus* (*S. hybrids*).

5. A recombinant DNA molecule containing a nucleic acid sequence according to any of the claims 1 to 4.

6. A recombinant DNA molecule according to claim 5 wherein the recombinant DNA molecule is a vector or a vector containing a promoter.

7. A host cells containing a DNA molecule according to claim 6.

8. A host cell according to claim 7 which is a bacterial cell, a yeast cell, an insect cell, a plant cell, or a mammalian cell.

9. A polypeptide encoded by a nucleic acid sequence according to any one of claims 1 to 4.

10. A polypeptide containing a portion or the full length amino acid sequence of SEQ ID NO:2 or derivatives thereof.

11. A polypeptide according to claim 10 wherein the polypeptide has flavone synthase II activity.

12. A polypeptide according to claim 11 wherein the polypeptide is derived from a plant of the group of plants consisting of gerbera (*Gerbera hybrids*), aster (*Callistephus chinensis*), snapdragon (*Antirrhinium majus*), chrysanthemum (*Chrysanthemum indicum*), dahlia (*Dahlia hybrids*), gloxinia (*Sinningia hybrids*), verbena (*Verbena hybrids*), and *Streptocarpus* (*S. hybrids*).

13. A transgenic plant containing a nucleic acid sequence according to any one of claims 1 to 4.

14. A transgenic plant according to claim 13 wherein the nucleic acid sequence is suitable for expression and wherein said expression optionally may be regulated or is developmentally regulated.

15. A transgenic plant according to any one of claims 13 or 14 selected from the group of plants consisting of gerbera (*Gerbera hybrids*), aster (*Callistephus chinensis*), snapdragon (*Antirrhinium majus*), chrysanthemum (*Chrysanthemum indicum*), dahlia (*Dahlia hybrids*), gloxinia (*Sinningia hybrids*), verbena (*Verbena hybrids*), and *Streptocarpus* (*S. hybrids*).

16. A method for the preparation of a transgenic plant having an altered flower color comprising introducing a nucleic acid sequence according to any one of claims 1 to 4 into a cell of a suitable plant and regenerating a transgenic plant from said cell and raising this transgenic plant over a suitable period of time and under conditions suitable for the expression of the nucleic acid sequence introduced.

17. A method according to claim 16 wherein the nucleic acid sequence introduced is expressed within the plant.

18. A method according to claim 16 or 17 wherein the transgenic plant is selected from the group of plants consisting of euphorbia (*E. pulcherrima*), cyclamen (*Cyclamen persicum*), rose (*Rosa hybrida*), pelargonium (*P. spec.*), begonia (*B. spec.*), carnation (*Dianthus caryophyllus*), and tulip (*Tulipa hybrids*).

19. A method according to claim 16 or claim 17 wherein the plant is capable of expressing an endogenous flavone synthase II (FNS II) which is coexpressed during expression of the nucleic acid sequence introduced.

20. A method according to claim 19 wherein the transgenic plant is selected from the group of plants consisting of gerbera (*Gerbera hybrids*), aster (*Callistephus chinensis*), snapdragon (*Antirrhinium majus*), chrysanthemum (*Chrysanthemum indicum*), dahlia (*Dahlia hybrids*), gloxinia (*Sinningia hybrids*), verbena (*Verbena hybrids*), and *Streptocarpus* (*S. hybrids*).

21. A method according to claim 19 or claim 20 wherein the endogenous flavone synthase II (FNS II) activity is reduced by the introduction of the nucleic acid sequence.

22. The use of a polypeptide according to any one of claims 9 to 12 for flavone synthesis.

23. The use according to claim 22 wherein the flavones are used as a medicament.

24. The use according to claim 23 wherein the flavones are used in cancer therapy.

25. The use according to claim 22 wherein the flavones are employed as bioactive substances.

Application number/ Numéro de demande : DE2000 0012/4

Documents of poor quality scanned
(request original documents in File Prep. Section on the 10th floor)

Documents de piètre qualité numérisés
(Pour obtenir les documents originaux, veuillez vous adresser à la Section de préparation des dossiers, située au 10e étage)

FIGURE 1A

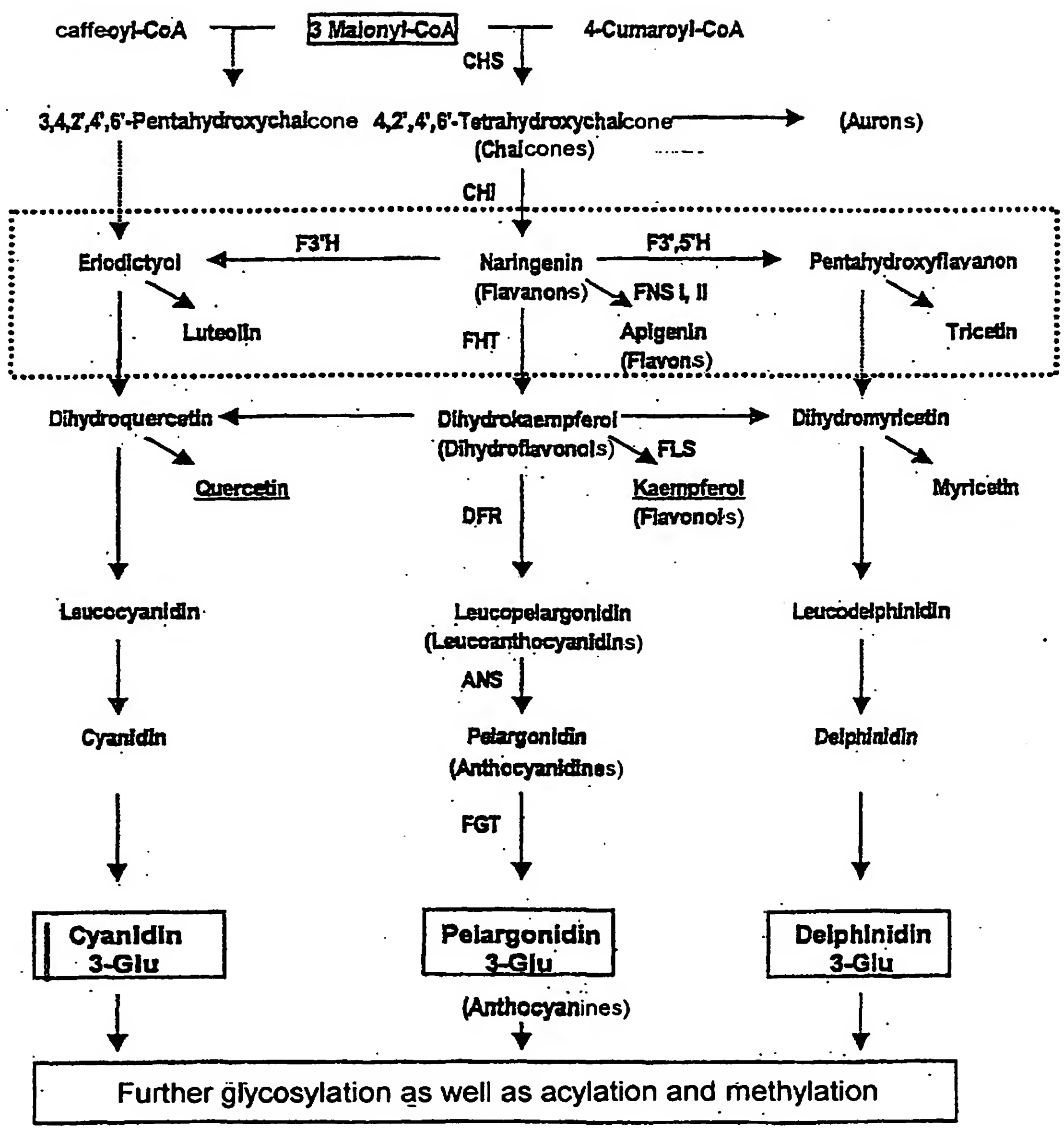
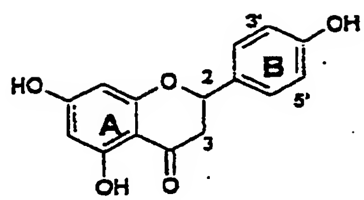
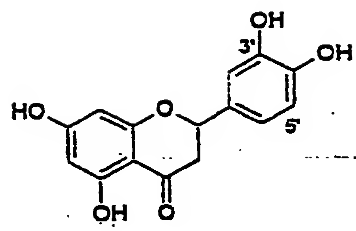

caffeoyl-CoA
3 Malonyl-CoA
4-Cumaroyl-CoA
CHS
3,4,2',4',6'-Pentahydroxychalcone
4,2',4',6'-Tetrahydroxychalcone
(Chalcones)
(Aurons)
CHI
Eriodictyol
F3'H
Naringenin
(Flavanons)
F3',5'H
Pentahydroxyflavanon
FNS I, II
Luteolin
FHT
Apigenin
(Flavons)
Tricetin
Dihydroquercetin
Dihydrokaempferol
(Dihydroflavonols)
FLS
Dihydromyricetin
Quercetin
Kaempferol
(Flavonols)
Myricetin
DFR
Leucocyanidin
Leucopelargonidin
(Leucoanthocyanidins)
Leucodelphinidin
ANS
Cyanidin
Pelargonidin
(Anthocyanidines)
Delphinidin
FGT
Cyanidin 3-Glu
Pelargonidin 3-Glu
Delphinidin 3-Glu
(Anthocyanines)
Further glycosylation as well as acylation and methylation

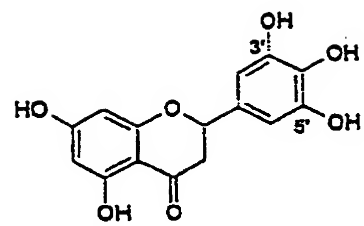
FIGURE 1B



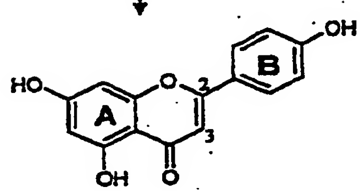
Naringenin



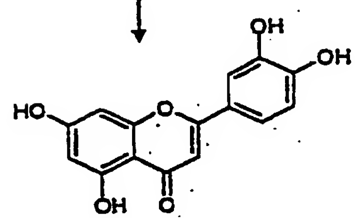
Eriodictyol



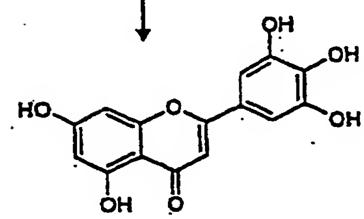
Pentahydroxyflavanon



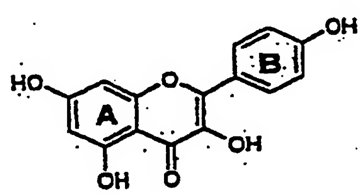
Apigenin



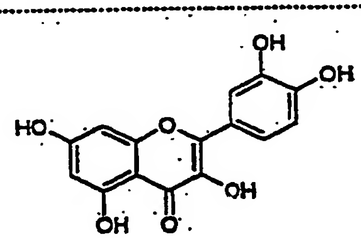
Luteolin



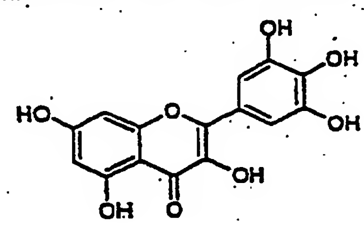
Tricetin



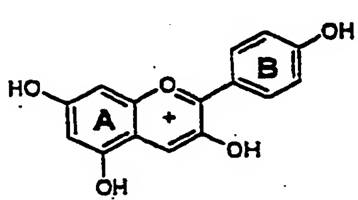
Kaempferol



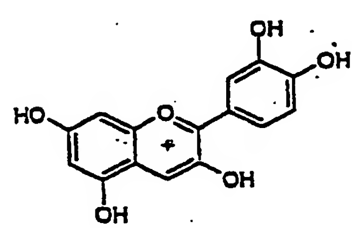
Quercetin



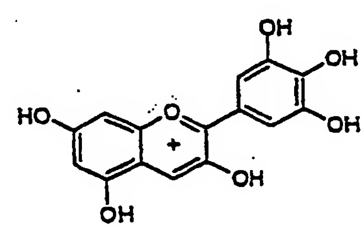
Myricetin



Pelargonidin



Cyanidin



Delphinidin

FIGURE 1C

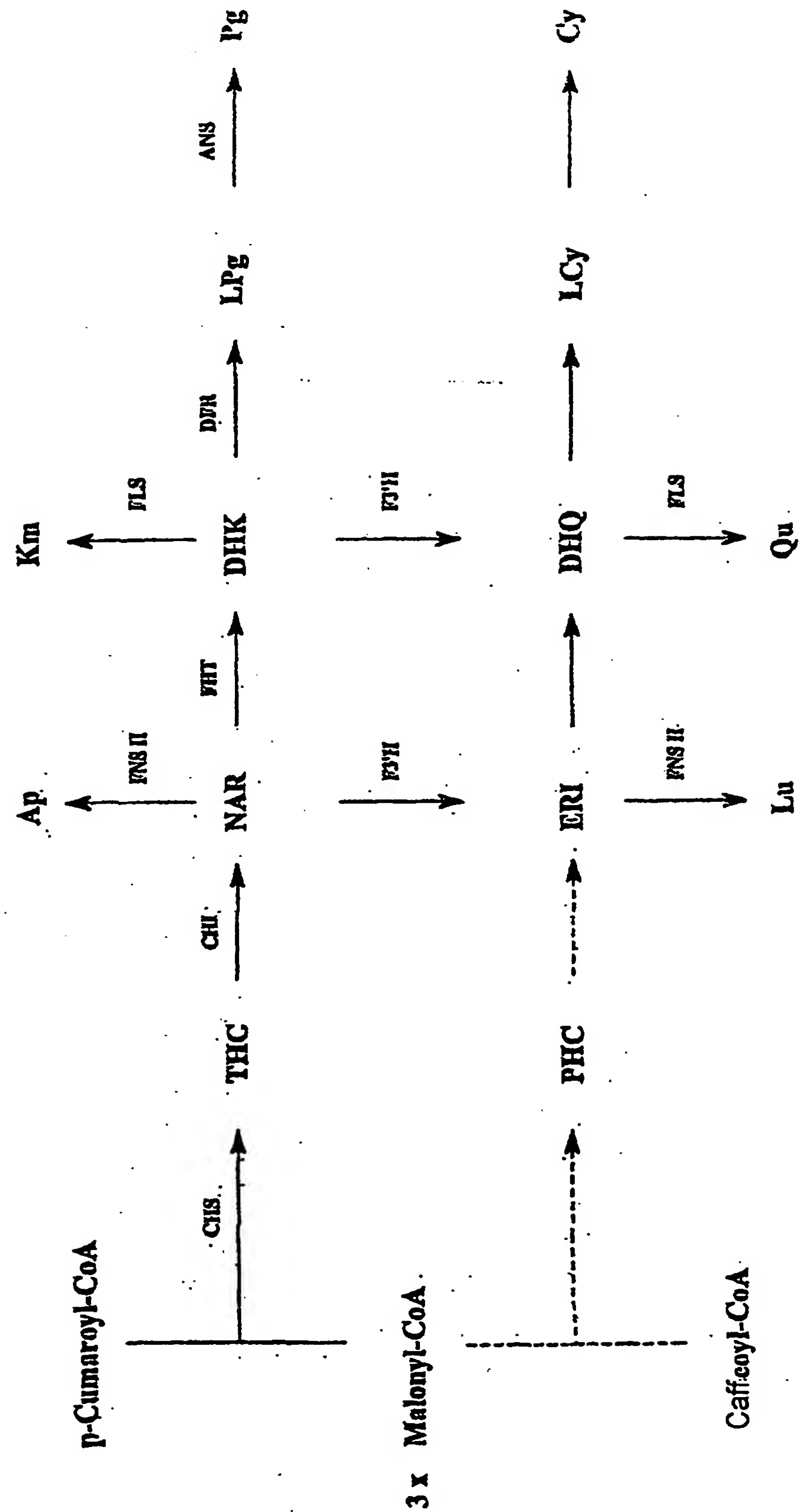
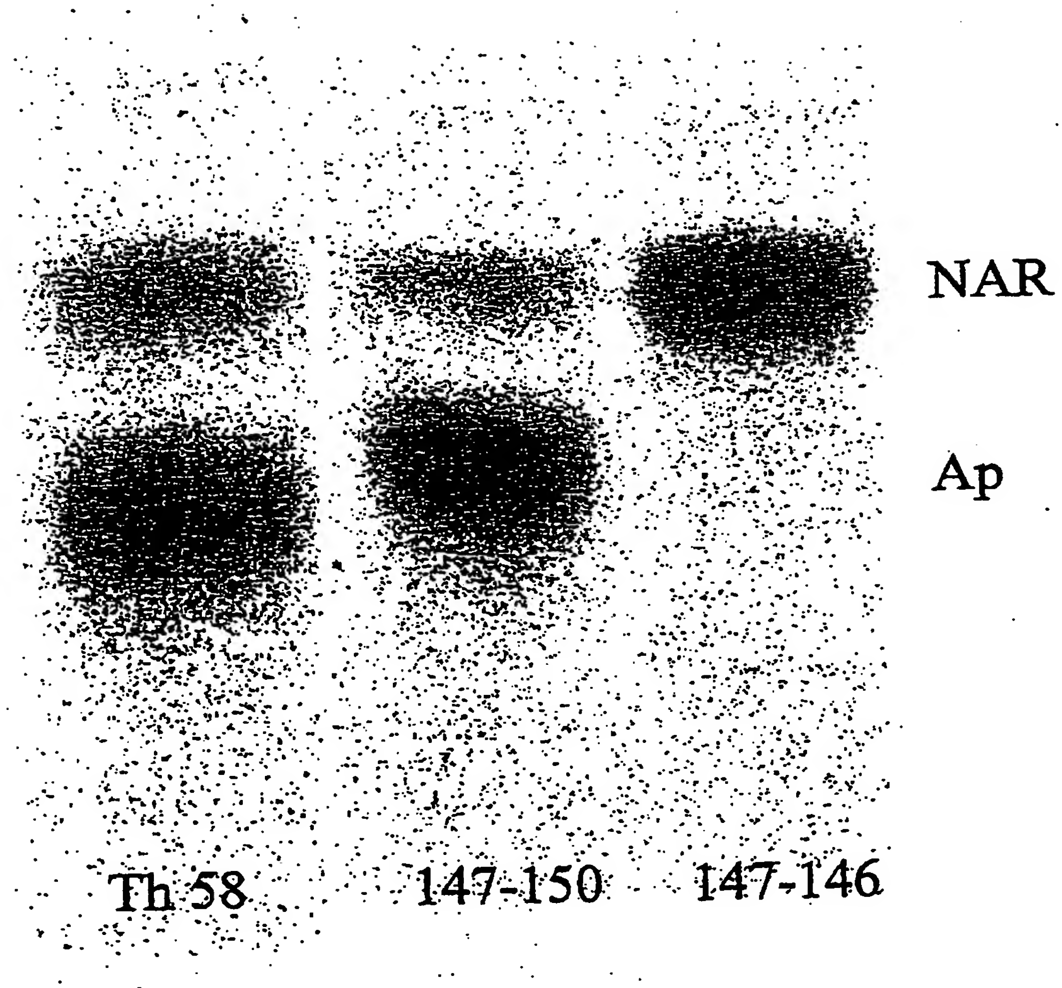

p-Cumaroyl-CoA
3 x Malonyl-CoA
Caffeoyl-CoA
CHS
THC
PHC
CHI
NAR
ERI
FNS II
Ap
Lu
F3'H
FHT
DHK
DHQ
FLS
Km
Qu
DFR
LPg
LCy
ANS
Pg
Cy

FIGURE 2



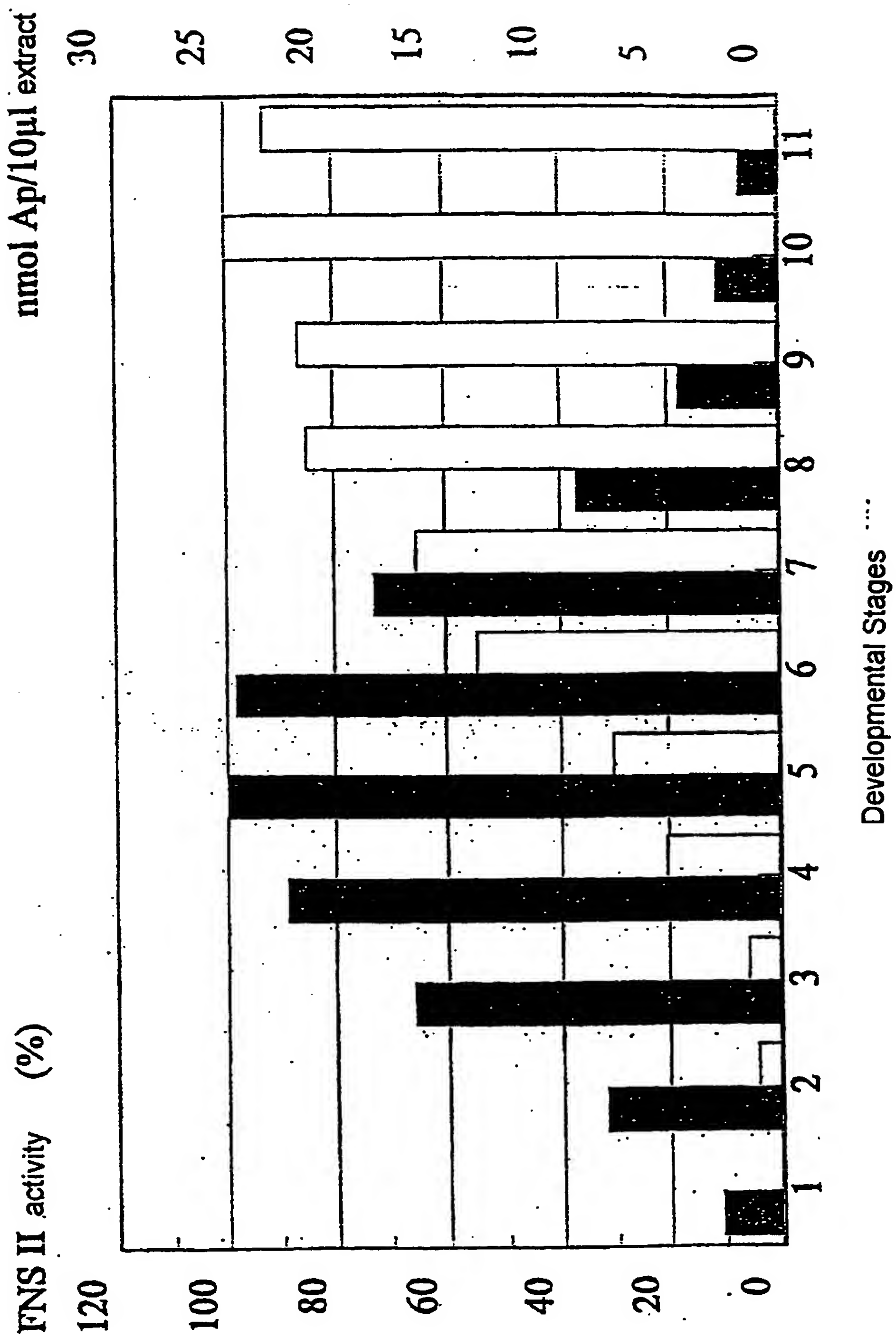

FNS II activity (%)
120
100
80
60
40
20
0
nmol Ap/10µl extract
30
25
20
15
10
5
0
1
2
3
4
5
6
7
8
9
10
11
Developmental Stages

FIGURE 3

FIGURE 4A

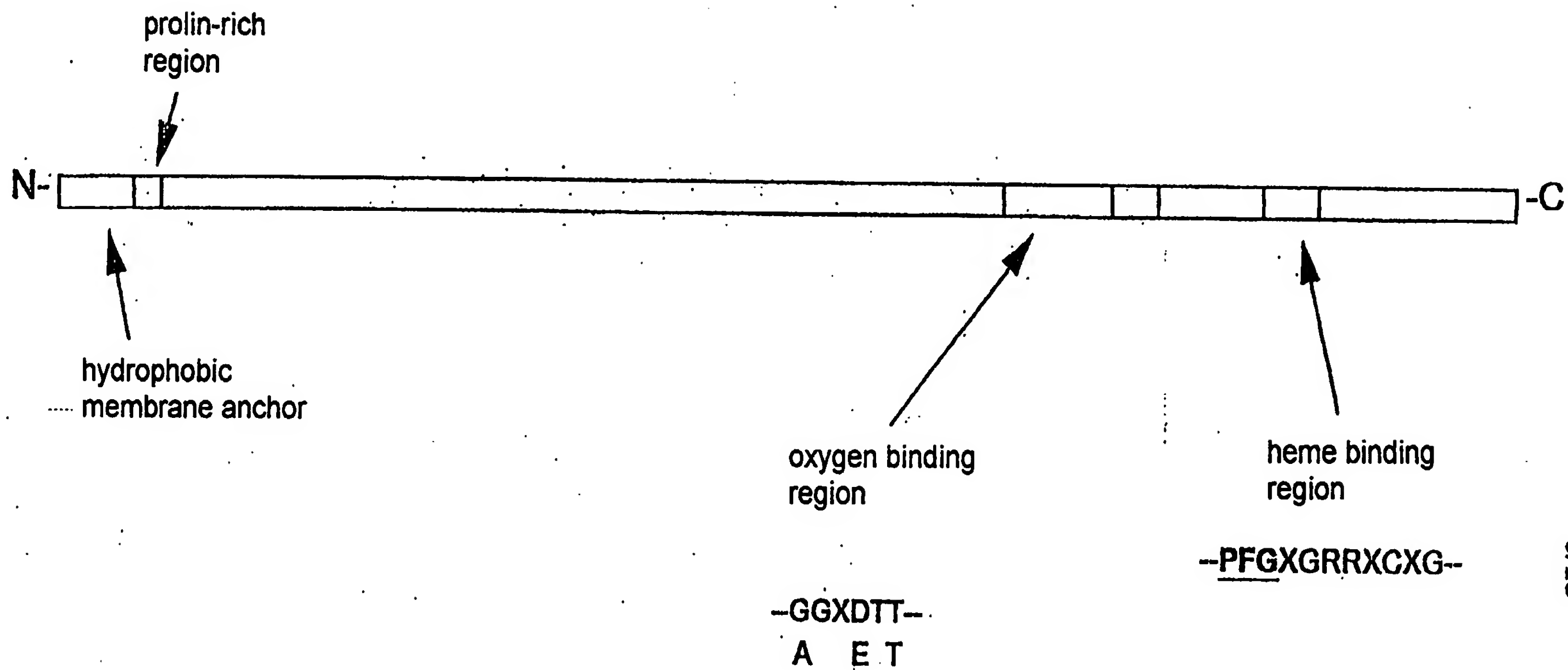
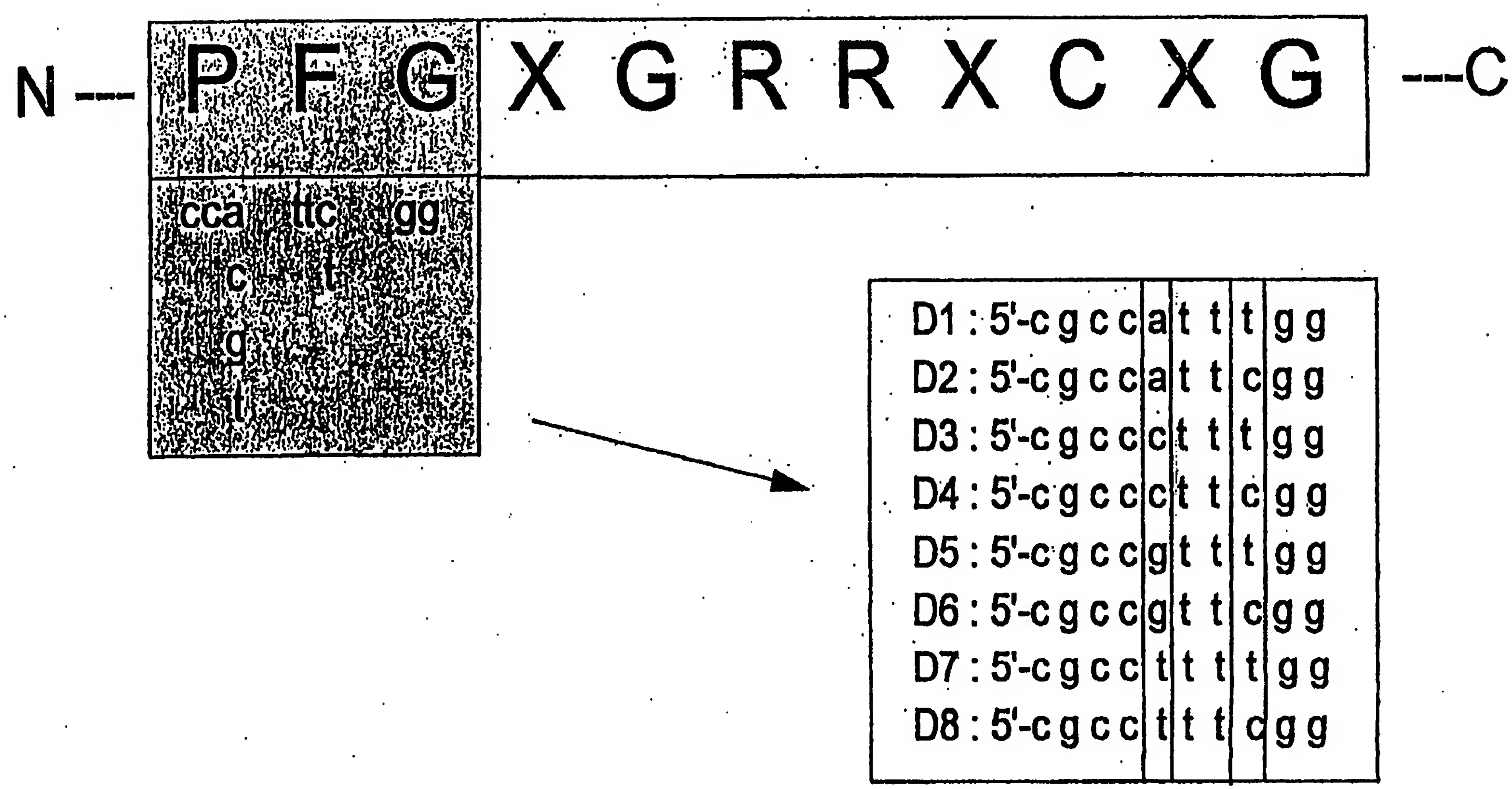

prolin-rich region
N-
-C
hydrophobic membrane anchor
oxygen binding region
heme binding region
--PFGXGRRXCXG--
--GGXDTT--
A E T

FIGURE 4B

Conserved region in the heme binding region



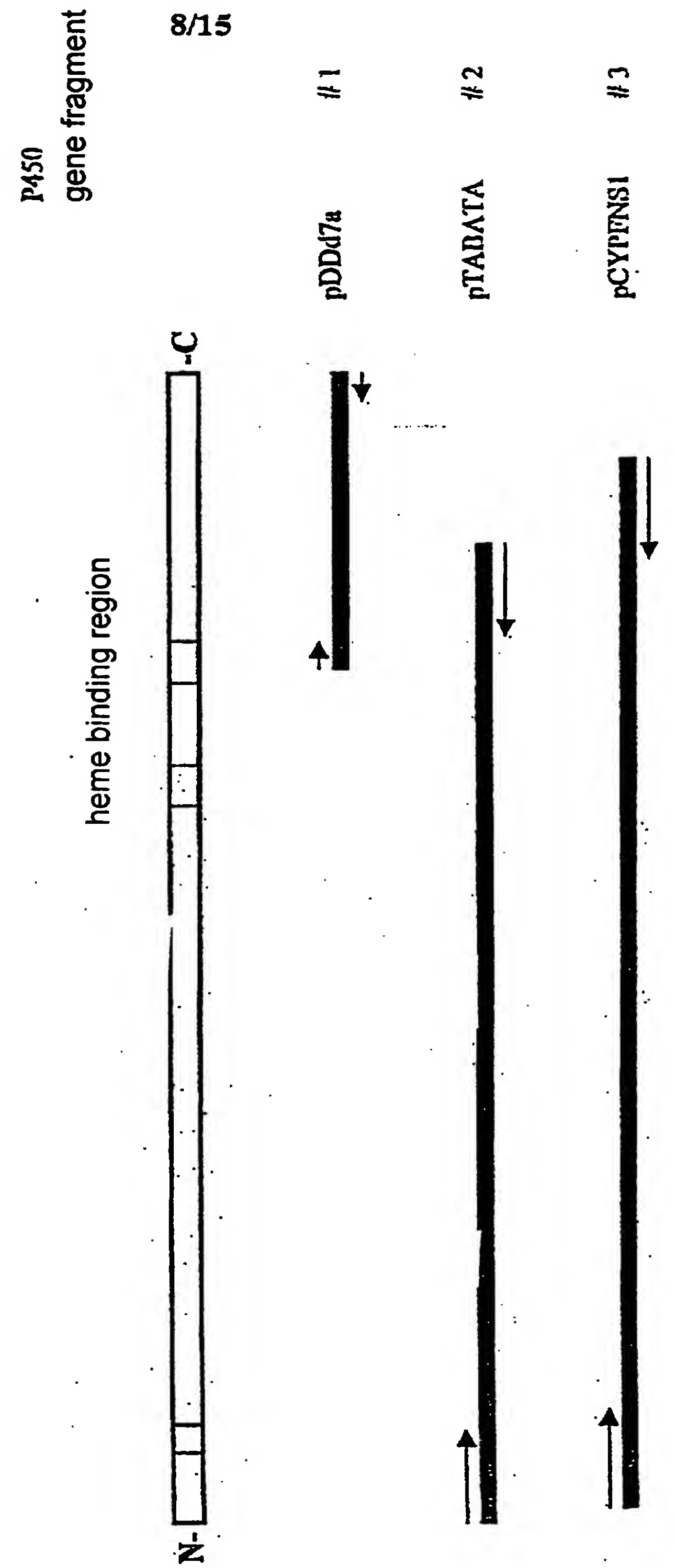

P450
gene fragment
heme binding region
N-
-C
pDDd7a
#1
pTABATA
#2
pCYPFNS1
#3

FIGURE 5

FIGURE 6

ATGTCCTAAC ACAACCCAAC ACCATGAATA CACTCCAACT CATCTTCCTC CTCTTCTTCT 60
TCCCAACCTT ACTCTTCCTC TACTGTCTCC CCTACAAAAG AAACCAAAAC CACCGCCGTC 120
TTCCGCCGTC CCCGCCATCT TTTCCGATCA TCGGCCACCT CCACCATCTC GGCCCACTCA 180
TCCACCAATC CTTCCACGCT CTCTCCACTC GCTACGGCTC TCTAATCCAC CTCCGTCTCG 240
GCTCAGTCCC ATGCGTCGTC GTTTCAACCC CAGACCTCGC CAAAGACTTC CTCAAAACAA 300
ACGAACTCGC GTTCTCATCA AGAAAACACT CCTTAGCCAT CGACCACATC ACCTATGGCG 360
TAGCATTTGC ATTCGCACCA TATGGAACTT ACTGGAAGTT CATCAAGAAA CTCTTCACAG 420
TGGAGCTTTT GGGCACCCAG AATCTCAGCC ATTTCCTACC CATTCGAACC CATGAAATTC 480
GCGAGCTTCT TCGAACGTTA ATGGTGAAAT CTAGGGCAAA GGAGAGAGTA AACTTGACGG 540
AAGAGTTGTT GAAGTTGACC AACAATGTGA TAAGTCAAAT GATGATGAGC ATTAGGTGTT 600
CGGGGACGAA TAGTGAGGCT GATGAAGCAA AGAATCTTGT TCGGGAAGTG ACCAAAATTT 660
TTGGACAGTT TAATGTTTCA GATTTCATAT GGTTTTGTAA GAACATAGAT TTGCAAGGGT 720
TTAAGAAGAG GTACGAGGGT ACACATAGAA GATATGATGC TTTGCTTGAA AGGATTATAA 780
TGGGGAGGGA AGAAAATAGA AGAAGAGGGA AGATAAAAGA TGGTGAAGGG AAAGATTTTC 840
TTGATATGTT ACTTGATGTT TTGGAGGATG GTAAGGCAGA GATTAAAATT ACTAGAGACC 900
ACATCAAAGC CTTGATTTTG GACTTTCTTA CAGCTGGGAC GGATACCACC GCGATTGCAA 960
TTGAATGGGC ACTAGTCGAA TTGATAAACA ACCCGAACGC TCTCGAGAAA GCAAGACAAG 1020
AGATTGATCA GGTCATCGGT GATGAGAGGC TAGTTCAAGA ATCAGACACG CCTAACCTCC 1080
CTTATATCCA AGCTATCATA AAGGAAGCCC TACGACTTCA CCCACCAATC CCAATGTTGA 1140
TTCGCAAGTC AACAGAAAAT GTAATTGTTC AGGGGTATGA CATCCCAGCC GGCACCTTGT 1200
TGTTTGTCAA TATTTGGTCC ATTGGAAGAA ACCCTCAATG TTGGGAAACC CCTTTAGAGT 1260
TCAAGCCTCA TCGGTTTTTG GATGGTGGTG ACCTTAAAAG CTCTTTAGAT ATTAAAGGCC 1320
ACAATTTTCA ACTATTGCCT TTTGGGACGG GGAGGAGAGG GTGTCCTGGT GTTAATTTGG 1380
CCATGAGAGA ACTCTCAGTG GTGATTGCAA ACCTCATACA ATGCTTTGAT TGGGATGTTG 1440
TAGGTGAACG ACTATTGAAT ACAGATGAAC GTGCTGGATT GACGGCTCCA AGGGCGGTAG 1500
ATTTTGTGTG TGTTCCATTG GAACGAGGAA ACACTTTGAA GATTCTTGGT TCAAACTAAA 1560
TTTATTTGTT GTTGCTTTCT TGATGGCAGT CGGTCTATCT ATAGGTCATA ATACCTTGGG 1620
ACTCACGTGT TTGAATCTTA ATACGCTTTT AGTACATTGC TTATCGTATA TCTTGGGTAT 1680
GCATGAAAAA AAAAAAA

FIGURE 7

Met	Asn	Thr	Leu	Gln	Leu	Ile	Phe	Leu	Leu	Phe	Phe	Phe	Pro	Thr	Leu
				5					10					15	
Leu	Phe	Leu	Tyr	Cys	Leu	Pro	Tyr	Lys	Arg	Asn	Gln	Asn	His	Arg	Arg
			20					25					30		
Leu	Pro	Pro	Ser	Pro	Pro	Ser	Phe	Pro	Ile	Ile	Gly	His	Leu	His	His
		35					40					45			
Leu	Gly	Pro	Leu	Ile	His	Gln	Ser	Phe	His	Ala	Leu	Ser	Thr	Arg	Tyr
	50					55					60				
Gly	Ser	Leu	Ile	His	Leu	Arg	Leu	Gly	Ser	Val	Pro	Cys	Val	Val	Val
65					70					75					80
Ser	Thr	Pro	Asp	Leu	Ala	Lys	Asp	Phe	Leu	Lys	Thr	Asn	Glu	Leu	Ala
				85					90					95	
Phe	Ser	Ser	Arg	Lys	His	Ser	Leu	Ala	Ile	Asp	His	Ile	Thr	Tyr	Gly
			100					105					110		
Val	Ala	Phe	Ala	Phe	Ala	Pro	Tyr	Gly	Thr	Tyr	Trp	Lys	Phe	Ile	Lys
		115					120					125			
Lys	Leu	Phe	Thr	Val	Glu	Leu	Leu	Gly	Thr	Gln	Asn	Leu	Ser	His	Phe
	130					135					140				
Leu	Pro	Ile	Arg	Thr	His	Glu	Ile	Arg	Glu	Leu	Leu	Arg	Thr	Leu	Met
145					150					155					160
Val	Lys	Ser	Arg	Ala	Lys	Glu	Arg	Val	Asn	Leu	Thr	Glu	Glu	Leu	Leu
				165					170					175	
Lys	Leu	Thr	Asn	Asn	Val	Ile	Ser	Gln	Met	Met	Met	Ser	Ile	Arg	Cys
			180					185					190		
Ser	Gly	Thr	Asn	Ser	Glu	Ala	Asp	Glu	Ala	Lys	Asn	Leu	Val	Arg	Glu
		195					200					205			

FIGURE 7

Val	Thr	Lys	Ile	Phe	Gly	Gln	Phe	Asn	Val	Ser	Asp	Phe	Ile	Trp	Phe
	210					215					220				
Cys	Lys	Asn	Ile	Asp	Leu	Gln	Gly	Phe	Lys	Lys	Arg	Tyr	Glu	Gly	Thr
225					230					235					240
His	Arg	Arg	Tyr	Asp	Ala	Leu	Leu	Glu	Arg	Ile	Ile	Met	Gly	Arg	Glu
				245					250					255	
Glu	Asn	Arg	Arg	Arg	Gly	Lys	Ile	Lys	Asp	Gly	Glu	Gly	Lys	Asp	Phe
			260					265					270		
Leu	Asp	Met	Leu	Leu	Asp	Val	Leu	Glu	Asp	Gly	Lys	Ala	Glu	Ile	Lys
		275					280					285			
Ile	Thr	Arg	Asp	His	Ile	Lys	Ala	Leu	Ile	Leu	Asp	Phe	Leu	Thr	Ala
	290					295					300				
Gly	Thr	Asp	Thr	Thr	Ala	Ile	Ala	Ile	Glu	Trp	Ala	Leu	Val	Glu	Leu
305					310					315					320
Ile	Asn	Asn	Pro	Asn	Ala	Leu	Gln	Lys	Ala	Arg	Gln	Glu	Ile	Asp	Gln
				325					330					335	
Val	Ile	Gly	Asp	Glu	Arg	Leu	Val	Gln	Gln	Ser	Asp	Thr	Pro	Asn	Leu
			340					345					350		
Pro	Tyr	Ile	Gln	Ala	Ile	Ile	Lys	Glu	Ala	Leu	Arg	Leu	His	Pro	Pro
		355					360					365			
Ile	Pro	Met	Leu	Ile	Arg	Lys	Ser	Thr	Glu	Asn	Val	Ile	Val	Gln	Gly
	370					375					380				
Tyr	Asp	Ile	Pro	Ala	Gly	Thr	Leu	Leu	Phe	Val	Asn	Ile	Trp	Ser	Ile
385					390					395					400
Gly	Arg	Asn	Pro	Gln	Cys	Trp	Glu	Thr	Pro	Leu	Glu	Phe	Lys	Pro	His
				405					410					415	

FIGURE 7

Arg	Phe	Leu	Asp	Gly	Gly	Asp	Leu	Lys	Ser	Ser	Leu	Asp	Ile	Lys	Gly
			420					425					430		
His	Asn	Phe	Gln	Leu	Leu	Pro	Phe	Gly	Thr	Gly	Arg	Arg	Gly	Cys	Pro
		435					440					445			
Gly	Val	Asn	Leu	Ala	Met	Arg	Glu	Leu	Ser	Val	Val	Ile	Ala	Asn	Leu
	450					455					460				
Ile	Gln	Cys	Phe	Asp	Trp	Asp	Val	Val	Gly	Glu	Arg	Leu	Leu	Asn	Thr
465					470					475					480
Asp	Glu	Arg	Ala	Gly	Leu	Thr	Ala	Pro	Arg	Ala	Val	Asp	Phe	Val	Cys
				485					490					495	
Val	Pro	Leu	Glu	Arg	Gly	Asn	Thr	Leu	Lys	Ile	Leu	Gly	Ser	Asn	
			500					505					510		

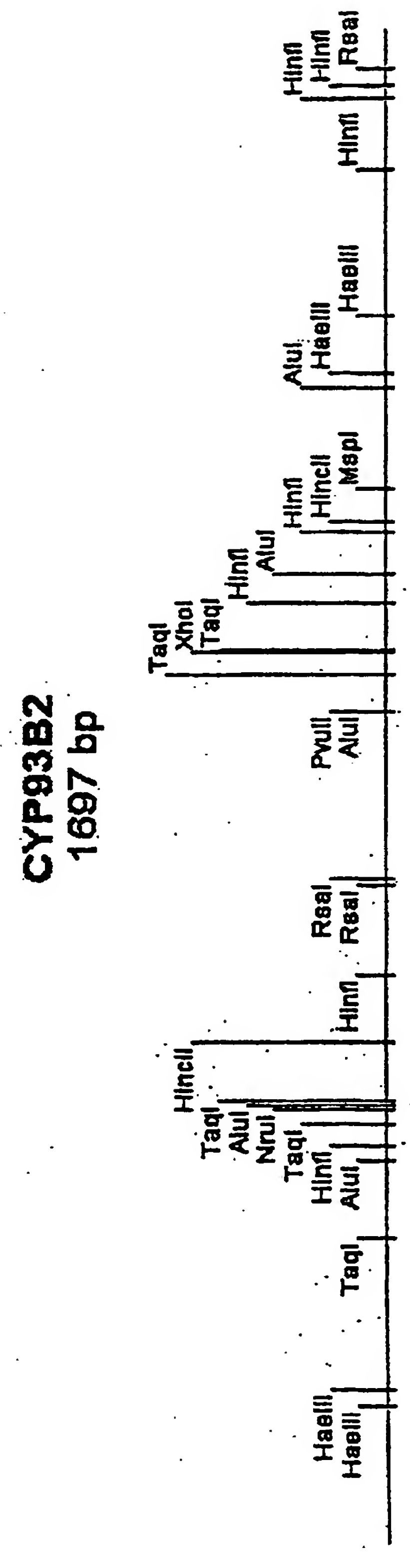
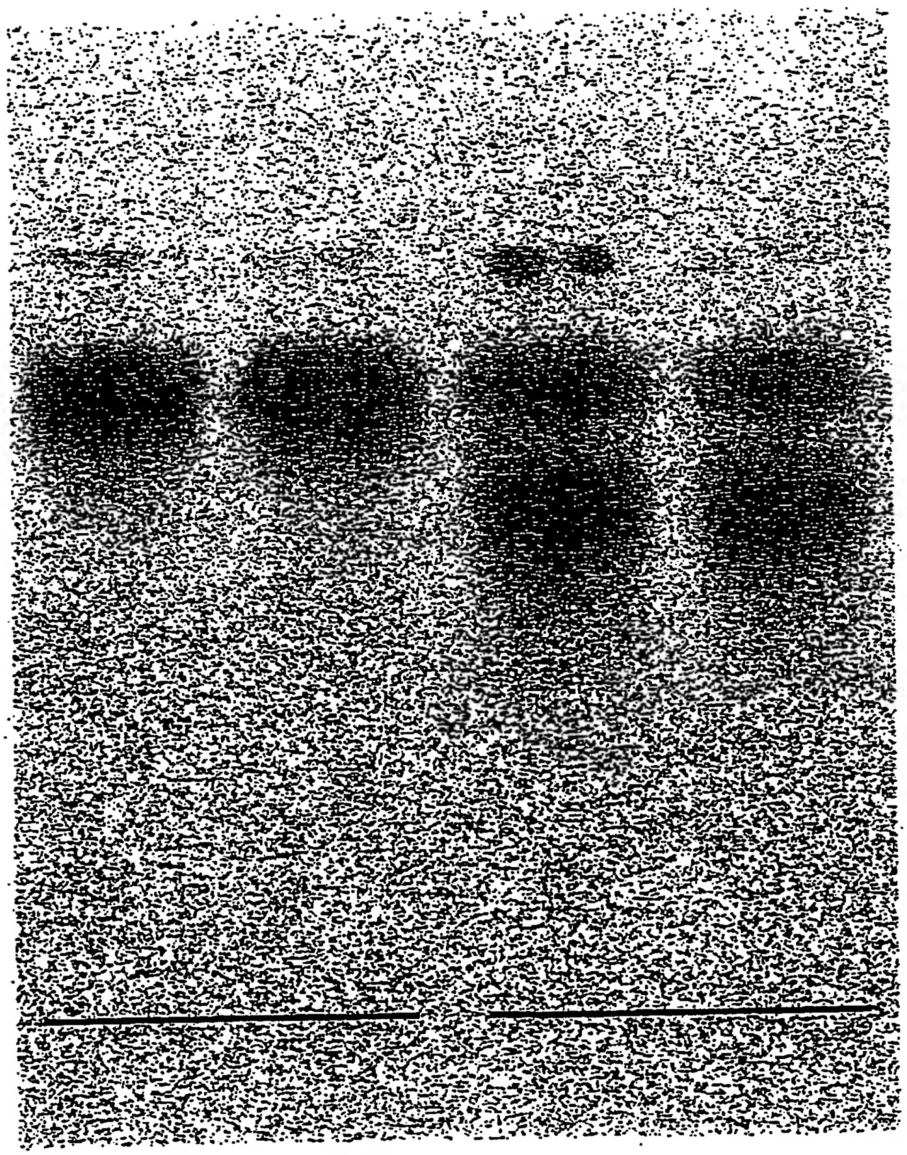

CYP93B2
1697 bp
HaeIII
HaeIII
TaqI
HincII
TaqI
AluI
NruI
TaqI
HinfI
AluI
HinfI
RsaI
RsaI
PvuII
AluI
TaqI
XhoI
TaqI
HinfI
AluI
HinfI
HincII
MspI
AluI
HaeIII
HaeIII
HinfI
HinfI
HinfI
RsaI

FIGURE 8

FIGURE 9



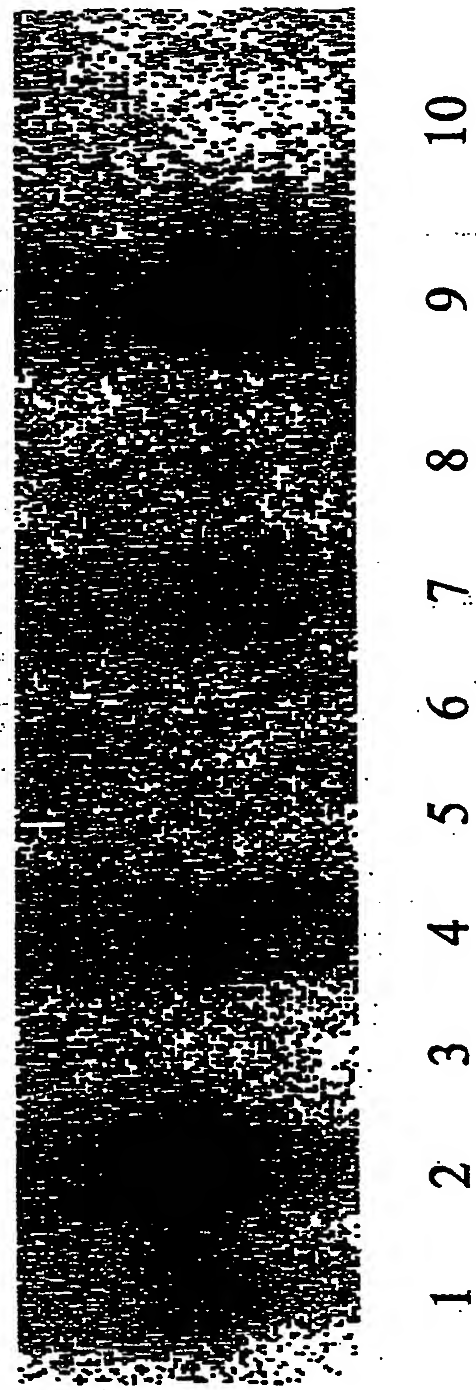


FIGURE 10